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VOL. XXXV. No. 6] [DECEMBER, 1954

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

UNIVERSITY OF MICHIGAN
FEB 2 1955
MEDICAL

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136 GOWER ST., W.C.1

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'The British Journal of Experimental Pathology.' Registered Office: 120 Moorgate, London, E.C.2

Annual Subscription, January to December, post free, Four Pounds net
Single Numbers, Sixteen Shillings net

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

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Date of Publication.—The Journal is published bi-monthly, in the middle of February, April, June, August, October, and December.

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Made and printed in Great Britain.

Vol. XXXV, No. 5 (October, 1954) was issued on 25.x 54.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XXXV DECEMBER, 1954 NO. 6

RHEUMATIC-LIKE LESIONS IN THE GUINEA-PIG: A CORRELATION OF TOXIC, ANAPHYLACTOGENIC, ARTHROPATHIC AND CHEMICAL PROPERTIES OF CERTAIN CRUDE POLYSACCHARIDES FROM *KLEBSIELLA PNEUMONIAE* TYPE B.*

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Received for publication June 2, 1954.

CERTAIN mucopolysaccharides from gastric mucin and a Friedlander's-type organism were found in prior experiments to produce joint and cardiovascular lesions in the guinea-pig (Jones, 1952; Jones and Carter, 1954). The ability of the polysaccharides to produce anaphylaxis had no correlation with the capacity to induce tissue lesions. Many substances in addition to the crude mucopolysaccharides have been found to induce cardiac-valvular lesions, but only the mucopolysaccharides seem to have the capacity to produce synovial proliferation, with or without exudate.

The present study deals with an extension of three observations, using crude polysaccharides derived by various techniques from a known organism, *Klebsiella pneumoniae* type B. The purpose of these experiments was : (1) to determine if anaphylactogenic capacity was necessary for production of joint lesions; (2) to perform exploratory chemical studies which might direct future research for the active "arthropathic" component or property. Thus far, refined capsular and somatic polysaccharides have not been used. Three of the crude mucopolysaccharides were selected for comparison of the morphologic effects; one of the polysaccharides was derived by acid hydrolysis and possessed toxic and anaphylactogenic properties; the other two polysaccharides were derived by alkaline hydrolysis and were non-toxic and non-anaphylactogenic.

MATERIALS AND METHODS.

Extraction of polysaccharides.

Kl. pneumoniae type B was grown on agar plates containing 4 per cent Bacto-peptone, 1 per cent glucose, and 0·0001 per cent dibasic sodium phosphate (Hoogerheide, 1939). After incubating for 18 hr. at 37°, the organisms were scraped gently from the agar and sus-

* This investigation was supported, in part, by a research grant (H1438), from the National Heart Institute of the National Institutes of Health, Public Health Service, U.S.A.

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pended in distilled water. For the extraction of two of the polysaccharides the technique of Wong (1938) was employed. The product derived by hydrolysis with acetic acid is termed " acid-hydrolysis polysaccharide " ; the other polysaccharide obtained by alkaline hydrolysis is termed " alkaline-hydrolysis polysaccharide ". Polysaccharides were extracted by two additional techniques, one by an alkaline-hydrolysis method (Meyer, Smyth and Palmer, 1937), and the other by formamide treatment (Fuller, 1938).

Chemical studies.

The partial chemical analyses of these polysaccharides are given in Table I. The acetyl determination is by the method of Elek and Harte (1936), but using the original phenolphthalein-alkaline solution (Pregl and Soltys, 1929) instead of the iodine solution. Hexosamine was determined by the technique of Elson and Morgan (1933), hexuronic acids by the procedure of Dische (1947), total nitrogen by Kjeldahl and protein by the biuret method. The polysaccharides were hydrolyzed, and sugars identified by paper chromatography using n-butanol-acetic acid and 4 : 1 phenol-water solvents with colour development by aniline-phthalic acid. Total reducing sugar after 10-hr. acid hydrolysis (Dagley and Dawes, 1949) was by the procedure of Somogyi-Shaffer-Hartman. Organic sulphate content was less than 0·1 per cent. Similar determinations were made on the discarded non-polysaccharide fractions obtained during the extraction procedures.

Sensitivity and toxicity studies.

Sensitization was carried out in guinea-pigs by the subcutaneous injection of 1 mg. of the polysaccharides per day for five consecutive days; on the fourteenth day after the first injection, animals were challenged with an intravenous (ear vein) injection of varying doses. Studies for cross-sensitization were also carried out (Table II).

Non-sensitized controls were given comparable intravenous injections and from this the LD_{50} value was determined. Animals were observed carefully during the next 24 hr. for such changes as hunched position, weakness, wheezing, cyanosis and time of death, and for such autopsy findings as visceral engorgement and ascites.

Morphologic studies.

The animals in the morphologic study were entirely separate from those used for the determination of the sensitivity. With each of two polysaccharides, an intravenous and a separate subcutaneous route was employed, but, with the third polysaccharide only the subcutaneous. Guinea-pigs, obtained from commercial breeders and weighing 180–240 g. were injected daily with 5 mg. of the polysaccharide if given subcutaneously, and 2·5 mg. intravenously. The polysaccharides were dissolved in 1 per cent isotonic sodium chloride solution and sterilized by autoclaving. When the subcutaneous route was used, animals were sacrificed after 3, 7, 14, 21 and 28 days. With the intravenous injections, animals were sacrificed at 3, 7 and 14 days ; normals as well as " pathologic " controls were employed. For the " pathologic " controls, 25 mg. of mucic acid and methylcellulose was given to some and to others benadryl with trypsin. All animals were maintained on Sperry Sure-rabbit green pellets, supplemented five times per week with leafy green vegetables. After sacrificing the animals, routine autopsy and microscopic studies were performed. The intact hearts were fixed in 10 per cent formalin ; after a single section, the exposed aortic and mitral valves were examined under the dissecting microscope. Micro-sections of the two paraffin-embedded blocks of the heart were made at three different levels and stained with toluidine blue and with haematoxylin and eosin. The brain and knee joints were examined in all animals. Care was taken to exclude the possibility of pneumonia and spontaneous streptococcal cervical lymphadenitis. Cervical lymph nodes were searched for at autopsy and microsections were made of any visibly enlarged nodes. No skin tests were performed for streptococcal sensitivity.

RESULTS.

The acid-hydrolysis polysaccharide alone possessed anaphylactogenic properties (Table II). This polysaccharide did not produce an anaphylactic response in animals given subcutaneous " sensitizing" injections of the other polysaccharides.

However, in those animals sensitized with killed bacteria anaphylaxis did occur with the higher challenging quantities of the acid-hydrolysis polysaccharides. This suggests that the toxic as well as anaphylactogenic properties of the killed bacteria may be present in the polysaccharide extracted by the acid-hydrolysis technique. Unfortunately, the elicitation of anaphylaxis by killed bacteria in polysaccharide-sensitized animals cannot be determined because of the marked respiratory and other symptoms produced by the intravenous introduction of whole organisms.

Compared to various proteins the antigenicity of the acid-hydrolysis polysaccharide is mild indeed; compared to the toxins from many other bacteria, this crude polysaccharide is relatively innocuous. The LD_{50} is approximately 0·067/mg./kg.; in other words, half of the guinea-pigs injected with 4 to 5 mg. of the acid-hydrolysis polysaccharide will die. The lethal effects of this acid-hydrolysis polysaccharide, administered intravenously, are not immediate but delayed, death usually occurring 5–18 hr. after the first injection. Animals become lethargic, have ruffled fur and a hunched position. At autopsy there is mild engorgement of the abdominal viscera, and a clear non-viscid fluid of mild to moderate quantity is found in the peritoneal cavity and occasionally in the pleural cavities. Sometimes there is a jelly-like mass in the retroperitoneal tissues. Adrenals are often engorged or haemorrhagic. Intussusception of the recto-sigmoid colon with prolapse of the haemorrhagic and necrotic tissue through the anus occasionally occurs during the first few days. Thus the acid-hydrolysis polysaccharide kills during the first day and from the first injection or, more rarely, from the effects of intussusception. In this sense, the animals which survive the first intravenous injection (Table III) represent a selected group resistant to the toxic effects of the polysaccharide.

Microscopically, in a few of the animals dying within the first day, there is a mild proliferation of the pericardial endothelium best seen at the reflection of the epicardium about the great vessels. An occasional fibrin thrombus is present in a sinus of Valsalva of the aortic valve. An appreciable percentage of the animals show mild synovial proliferation; occasionally this is accompanied by a slight fibrinous exudate in the joint space (Table III).

Joint changes appear by the third day and become worse with continued injections. The joint changes consist of synovial proliferation, especially at the recesses and at the meniscal-synovial junctures (Fig. 3). Some eosinophilic or fibrinous material may be found within the joint space (Fig. 4). The severest lesions are found with the alkaline-hydrolysis polysaccharide (Fig. 5–7), whether given intravenously or subcutaneously; these lesions appear by the second week. The proliferation of the synovial cells is quite marked and at times the fibrin in the joint space is abundant; the latter, in particular, is reminiscent of scorbutic arthropathy. There are some mononuclear leukocytes and fibroblastic cells in the periarticular areolar tissue. The epiphyseal lines are unchanged and there is no alteration in bone formation.

The cardiac-valvular and joint changes induced by the three polysaccharides are given in Table III. The cardiac-valvular lesions are present in many of the animals but are never severe (Fig. 1), and, as stated in previous reports (Jones, 1951; Jones and Carter, 1954), these are non-specific. The detailed description of the gross and histologic changes in the cardiac valves need not be repeated here.

The comparison of the toxic, pathologic, anaphylactogenic and chemical

properties permit several tentative conclusions : (1) The acid-hydrolysis polysaccharide is relatively toxic, animals dying from 5–18 hr. after the initial injection ; if the initial injection is survived, subsequent injections are not lethal. (2) There is no appreciable difference whether the material is given by the intravenous or subcutaneous route, except that the toxic effects are far more marked with the intravenous. (3) Cardiac-valvular as well as joint changes occur within three days after initiation of injections. With the acid-hydrolysis polysaccharide, some changes are seen within the first day. (4) The ability of the polysaccharides to produce the state of sensitivity in which anaphylaxis may be elicited does not appear to be necessary for the development of the joint and cardiac lesions, and does not appear to be related to the nitrogen or protein content of the substance used. (5) Different batches of the polysaccharides obtained by the same technique have considerable variation in protein, nitrogen, hexosamine, and hexuronic acid content (Table I), but possess the same toxic, anaphylactogenic or arthropathic properties. (6) The joint lesions are more severe in animals receiving the alkaline-hydrolysis compound which is non-anaphylactogenic and relatively non-toxic. (7) Some healing and diminution of the joint lesions appears by the third or fourth week of repeated subcutaneous injections.

DISCUSSION.

The cardiac-valvular lesions of the guinea-pig do not seem to be specific for any exogeneous agent but probably represent some form of a general inflammatory response (Jones and Carter, 1954) ; however, the joint changes thus far observed have been associated only with the injection of the mucopolysaccharides. None of these crude mucopolysaccharides has been identified as to any definite chemical

DESCRIPTION OF PLATES.

FIG. 1.—The mild endocardial proliferation of the mitral valve in this photograph was present in several areas, occurring only on the auricular aspect. Animal was given 5·0 mg. acid-hydrolysis polysaccharide (Wong) subcutaneously for 3 weeks. × 250.

FIG. 2.—About the coronary artery there is a grouping of fibroblastic and myocytic cells. Animal received alkaline-hydrolysis polysaccharide (Wong) subcutaneously for 1 week. There was only a mild synovial change in the knee joint of this animal. × 250.

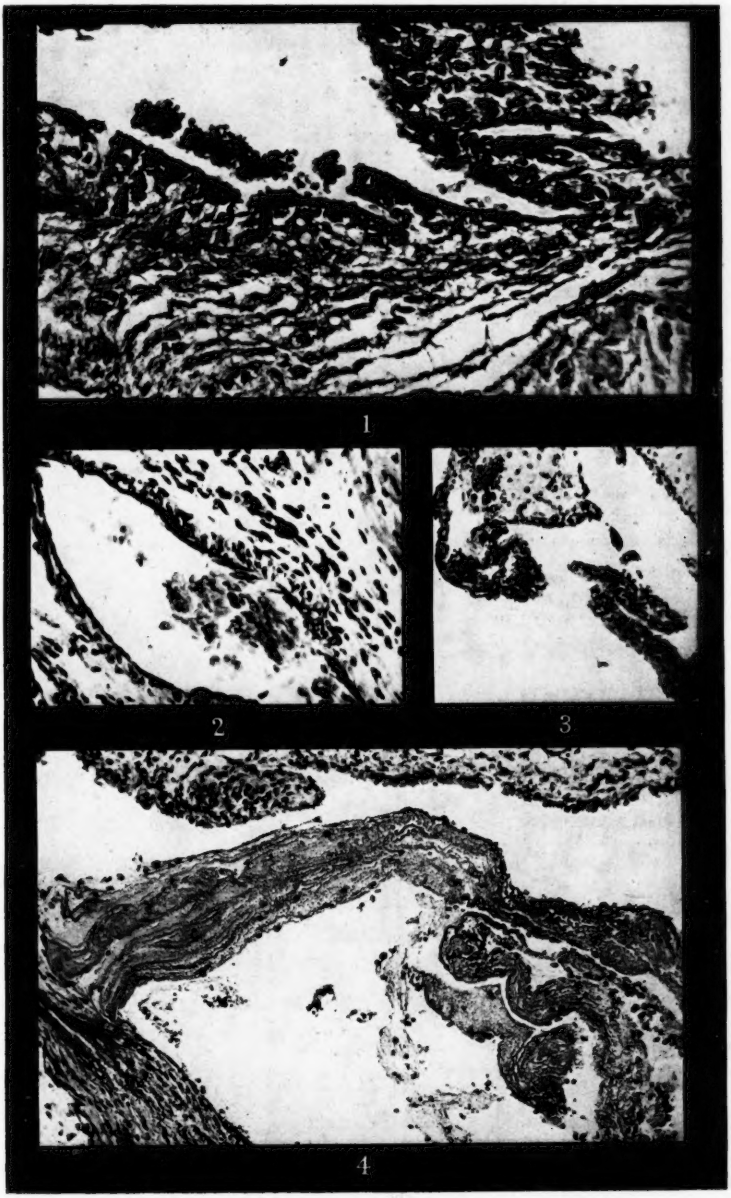
FIG. 3.—A mild proliferation of synovial cells is observed near the meniscus in the knee joint. No exudate was present in this joint. Animal received acid-hydrolysis polysaccharide (Wong) subcutaneously for 2 weeks. × 125.

FIG. 4.—A pannus of fibrinous exudate and a few mononuclear leukocytes extend into the joint space from the synovial recess. Above is a group of the proliferated synovial cells seen over many of the joint surfaces other than those of the articular cartilage. There are no changes in the epiphyseal areas suggestive of scurvy and alterations in the periarticular tissues are minimal. Animal received alkaline-hydrolysis polysaccharide (Wong) subcutaneously for 1 week, 5 mg. per day. × 125.

FIG. 5.—Extensive pannus formation is present in this knee joint. Proliferated synovial cells form the surface and fibroblasts and collagen compose the stroma. Within the joint space are some homogeneous basophilic material and some mononuclear leukocytes ; the latter appear to arise by shedding from the synovial surface. Fibroblasts, some collagen and basophilic ground substance are prominent in the periarticular area. Animal received alkaline-hydrolysis polysaccharide (Wong) subcutaneously for 4 weeks. × 125.

FIG. 6.—Some basophilic material clings to the pannus on proliferated synovium. Thin-walled vessels are present within the stroma of the pannus. This guinea-pig received alkaline-hydrolysis polysaccharide (Wong) subcutaneously for 3 weeks. × 125.

FIG. 7.—Higher magnification of Fig. 6, showing in greater detail the synovial cells, the smaller stromal fibroblasts of the pannus, the distended capillary channels and some of the granular material clinging to the synovial surface. × 250.



Jones, Carter and Rankin.

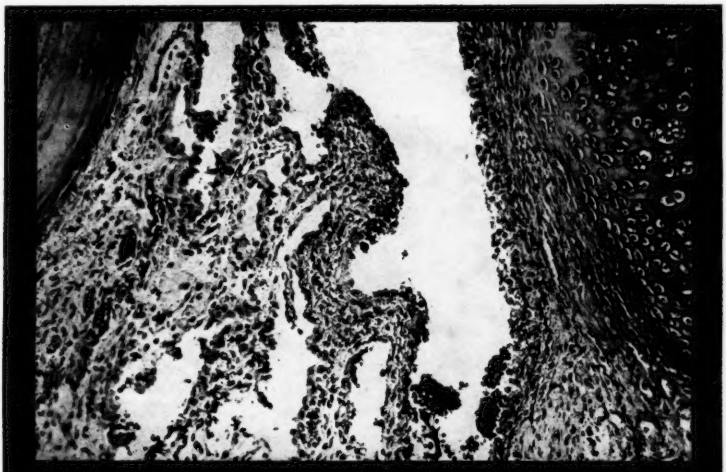


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TABLE I.—*Some Chemical Properties of Crude Polysaccharides of* Kl. pneumoniae *B.*

Extraction method.	Per cent content of												
	Biuret protein.	N.	Glucos-amine.	Hex-uronic acid.	Acetyl.	Reduc-ing sugar.	Gluc-uronic acid.	Glu-cose.	Rham-nose.	Lac-tose.	Ribose.	Galac-tose.
Formamide (Fuller)	38·8	3·02–9·2	0·62–2·38	7·2–19·3	5·24	18·9	+	+	+	+	+	+
Alk.-hydrolysis (Meyer)	0	1·0	2·25	18·5	4·36	44·0	+	+	+	+	0	0
Acid-hydrolysis (Wong)	3·4–15·5	0·8–3·2	0·38–1·50	10·0–19·2	1·18	39·8	+	+	+	+	0	+
Alk.-hydrolysis (Wong)	6·8–10·5	1·9–5·1	0·38–0·85	12·8–18·5	2·06	34·6	+	+	+	+	0	0

+ Qualitative identification by paper chromatography.

The range of values for polysaccharides obtained by separate extractions is given for protein, nitrogen, hexosamine and hexuronic acid ; values for acetate and reducing sugar are from pooled samples.

TABLE II.—*Production of Anaphylaxis in Animals Sensitized with the Same and with Different Polysaccharides of* Kl. pneumoniae.

		Challenge.										
		Dose (mg.).										
Sensitization.	Agent.	0·1.	0·5.	1·0.	2·0.	3·0.	4·0.	5·0.	7·0.	10.	15.	20.
Formamide (seitz filtered)	Same	0/1	0/1	0/7	—	0/2	—	—	—	—	—	—
Ditto	Wong acid-hydrolysis	—	—	0/3	0/2	—	—	0/2	—	—	—	—
Formamide (autoclaved)	Same	—	—	0/1	0/2	—	0/1	—	—	—	—	—
Wong acid-hydrolysis (autoclaved)	,,	—	—	1/2	3/3	4/4	2/2	8/8	1/1	1/3	—	—
Ditto	Formamide	—	—	—	—	—	0/1	0/2	—	—	—	—
,,	Killed bacteria	—	—	0/2	—	—	—	*/3	—	*/1	—	—
,,	Wong alkaline-hydrolysis	—	—	0/4	—	—	—	0/2	—	0/1	—	—
Wong alkaline-hydrolysis	Same	—	—	—	0/2	—	0/1	0/1	0/1	0/2	0/1	—
Ditto	Acid-hydrolysis	—	—	0/1	—	0/1	—	—	0/1	0/1	—	0/1
,,	Killed bacteria	—	—	0/2	—	—	—	*/2	—	*/2	—	—
Killed bacteria	Same	—	—	0/4	0/2	—	—	0/1	*/2	*/2	—	—
,,	Alkaline-hydrolysis polysaccharide	—	—	0/2	—	—	—	0/3	—	1/4	—	—
,,	Acid-hydrolysis	—	—	0/4	?/1	—	—	3/3	—	2/2	1/1	—
Meyer alkaline hydrolysis	Same	—	—	—	—	0/2	—	0/3	—	—	—	—
Ditto	Wong acid-hydrolysis	—	—	—	—	0/2	—	0/3	—	—	—	—

Numerator: number of animals reacting.
Denominator: ,, ,, tested.
* Response to bacteria obscures anaphylaxis.

TABLE III.—*Comparison of Cardiac-valvular and Joint Lesions Following the Daily Injection of* Kl. pneumoniae *Polysaccharides Extracted by Three Methods.*

	1 day.			3 days.			7 days.			14 days.			21 days.			28 days.		
	Cardiac valves.	Joints.		Cardiac valves.	Joints.		Cardiac valves.	Joints.		Cardiac valves.	Joints.		Cardiac valves.	Joints.		Cardiac valves.	Joints.	
Polysaccharide.		Synov. prolif.	Exu-date.		Synov. prolif.	Exu-date.		Synov. prolif.	Exu-date.		Synov. prolif.	Exu-date.		Synov. prolif.	Exu-date.		Synov. prolif.	Exu-date.
Acid-hydrolysis: subcutaneous 5·0 mg. per day	—	—	—	3/4	4/4	1/4	2/4	3/4	2/4	3/3	3/3	2/3	3/4	4/4	1/4	3/3	2/3	—
intravenous, 2·5 mg. per day	4/15*	3/15	0/15	4/5	5/5	3/5	4/5	5/5	3/5	3/5	4/5	1/5	—	—	—	—	—	—
Alkaline-hydrolysis: subcutaneous, 5·0 mg. per day	—	—	—	2/4	2/4	2/4	3/4	3/4	2/4	2/4	3/4	2/4	4/4	3/4	3/4	4/4	4/4	1/4
intravenous, 2·5 mg. per day	—	—	—	2/4	3/4	3/4	3/4	3/4	0/4	4/5	5/5	1/5	—	—	—	—	—	—
Meyer polysaccharide: subcutaneous, 5·0 mg. per day	—	—	—	0/4†	1/4	2/4	0/4	1/4	3/4	0/4	0	1/4	0/4‡	0	3/4	2/4	0	2/4

Numerator: number of animals showing changes.
Denominator: " " examined.

* An additional animal showed a small fibrin thrombus in a valsalval sinus of the aortic valve and two animals had scattered eosinophils and mononuclear leukocytes in the epicardium.
† Focal dense infiltration of mononuclear leukocytes in mitral valve.
‡ One focus of necrosis and polymorphonuclear infiltration in mitral valve.

or molecular structure such as those established for the pneumococcus type-specific (capsular) and somatic (heterophile antibody and species-specific) polysaccharides (Brown, 1939; Goebel, Shedlovsky, Lavin and Adams, 1943), or for the Friedlander type B capsular polysaccharide (Heidelberger, Goebel and Avery, 1925).

To pose the simplest causal relationship, the arthropathic action may be attributed to the localization of the polysaccharide (or one of its components or modifications) in the joint tissue. There is evidence from dye-tagged antibody studies that certain antigenic bacterial polysaccharides do reach the synovial membranes in considerable quantities and remain in various tissues of injected mice for well over a month (Hill, Deane and Coons, 1944). In contrast to the wide tissue distribution and long storage of the acidic macromolecules of Friedlander type B and pneumococcus types II and III, the smaller molecules of the neutral polysaccharides of group A streptococci are rapidly excreted (Schmidt, 1952). Other substances besides polysaccharides also enter joint spaces; foreign proteins in relatively high concentration have been identified by serologic means in aspirated joint fluid (Opie, 1924; Bennett and Shaffer, 1939). Presuming that many of the foreign substances, proteins, polysaccharides and so forth, injected into guinea-pigs in prior experiments (Jones and Carter, 1954) do reach the joint tissues or fluid, then the mucopolysaccharides are the only substances thus far tested which have an arthropathic effect.

The initial effusion into the peritoneal cavity with the acid-hydrolysis polysaccharide suggests an affinity of some crude polysaccharides for mesothelial surfaces. In the present interpretation the effusion is considered to be part of the " toxic " response but, of course, it is quite possible that an endotoxin possessed by many Gram-negative bacteria may be merely attached to or incorporated in the crude mucopolysaccharide with an affinity for mesothelial and synovial surfaces. Comparison of the toxic components of different Gram-negative organisms is not fruitful; the toxic component of the somatic antigen of *Shigella flexneri* may be a distinct and rather labile entity, independent of protein, lipid or carbohydrate components (Tal and Goebel, 1950); the toxicity of the somatic antigen of *Shigella shigae* is dependent upon the integrity of the undegraded molecules comprised of protein, phospholipid and carbohydrate fractions (Morgan and Partridge, 1940).

If ability to produce anaphylaxis is to be used as a criterion of anaphylactic hypersensitivity, then this form of sensitiveness could not have an important rôle in the joint changes. Furthermore, the lesions appear sooner than one would expect the tissue or circulating antibodies to develop. There is a possibility that the joint changes represent some type of tissue response related to the Shwartzman phenomenon or to the pre-anaphylactic cutaneous response which Dienes (1936) regards as the first manifestation of active immunization.

It should be emphasized that there are two aspects of such host-responses as toxicity, antibody formation, anaphylaxis and joint lesions—the nature of the exogenous agent and the " mechanism " of the host-responses. While some progress has been made in the chemical separation and identification of toxic, antigenic and haptenic components of certain bacteria, a comparable knowledge of the mechanism of response has not been achieved. Without further understanding of these host responses and their interrelationships, the pathogenesis of the joint lesions remains obscure.

SUMMARY.

Cardiac-valvular and joint lesions have been induced by the intravenous and by the subcutaneous injection of crude polysaccharides from *Klebsiella pneumoniae* type B. The cardiac-valvular lesions are mild and non-specific. The arthropathic effect of the polysaccharides is not attributable to toxic or anaphylactogenic properties or to differences in hexosamine, hexuronic acid, acetate, nitrogen, protein, mono- or di-saccharide content.

The authors are most grateful to Jean Applegate for technical assistance, to Perry Hunt for care of animals, to Dr. Arthur Frisch for helpful advice on bacterial polysaccharides and to Jim Phillips for photography.

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THE VIRULENCE OF STRAINS OF *BACTERIUM COLI* FOR MICE.

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Received for publication July 8, 1954.

ONE of the aspects of bacteriology which remains obscure in spite of a great volume of research is the precise nature and cause of virulence in micro-organisms. For a given host there are often very large variations in virulence between different strains of the same species. It is with these inter-strain differences in virulence that the present paper is concerned.

An essential property of virulent strains is their ability to grow in the host, whereas non-virulent strains are usually eliminated sooner or later by the host's defences. It seems that virulent organisms must be able to resist one or more of the defence reactions to which avirulent strains succumb. In this light the study of virulence becomes a study of host defence mechanisms and the variation of microbial response to them. Very little information at the molecular level is available on this subject, except for work relating virulence of various species to the possession of a capsule (Avery, 1932). In pneumococci the capsule is composed of polysaccharide material and prevents removal of the organisms by phagocytosis. Injection of bacteria together with mucin or gum tragacanth is well known to increase their apparent virulence and because of the diversity of substances which cause this effect (Olitzki, 1948), one might think that mere mechanical coating of bacteria was the important factor. Against this belief and pointing to a more specific effect is the fact that specific capsular antiserum to pneumococci renders them susceptible to phagocytosis in spite of the fact that the capsule appears even larger than before.

In the case of *Salmonella typhi* Felix and Pitt (1935) have produced evidence that virulence for mice and probably for man is associated with an unstable antigen, the virulence or Vi antigen. This antigen prevents normal O agglutination *in vitro* and it is believed by Felix to act *in vivo* by masking the reaction between normal serum antibodies of the O type and bacteria. Since this combination between normal serum antibodies and bacterial antigens is a necessary preliminary to complement-mediated lysis and also greatly enhances the efficiency of phagocytosis, prevention of combination would increase the virulence of the organism.

It is theoretically possible that some hosts are resistant to infection by certain bacteria because they do not possess all the growth factors and nutrients required by the organisms for growth (Bacon, Burrows and Yates, 1951). It is unlikely that variations in growth requirements can explain many of the observed differences in virulence among the enteric organisms since most strains of *Salmonella* and *Bacterium coli* have little or no organic requirements other than for glucose.

The observations that only 6 per cent of "normal" coliform organisms were inhibited by DL-serine, whereas 65 per cent of the various serological types of

Bact. coli isolated from cases of gastro-enteritis were susceptible to this inhibition (Rowley, 1953), suggested that this might be an *in vitro* reaction paralleling differences in virulence. A similar relationship between serine inhibition and human virulence has been reported by Dubos (1949) for *Mycobacterium tuberculosis*.

Although the relationship between mouse virulence and human pathogenicity may be in doubt (Miles, 1951), these studies, for obvious reasons, were carried out on mice. Twenty strains of *Bact. coli* were selected so as to include representatives of the serine-sensitive and -resistant groups, strains sensitive to other amino acid inhibitions and strains isolated from cases of gastro-enteritis.

MATERIALS AND METHODS.

Of the 20 *Bact. coli* strains, 6 were from a collection of normal faecal coliform organisms; of the others, 10, prefixed E, were kindly supplied by Dr. Joan Taylor and came from cases of gastro-enteritis; we are indebted to Dr. Studzinski of Glasgow for 3 others, prefixed A, belonging to serological type O 111 and also derived from cases of gastro-enteritis. All the strains were freeze-dried from a serum broth culture and 6 slopes of Dorset egg medium were inoculated from one of the freeze-dried tubes of each strain. The slopes were kept at 2° and after a slope had been opened 10 times it was rejected and a fresh slope used. In this way the culture used was always a second subculture from the master freeze-dried strain. All the strains would grow readily on a glucose inorganic salt medium containing ammonium salts as the sole nitrogen source (Davis and Mingioli, 1950). The biochemical reactions of the 20 strains, together with their amino acid sensitivities and antigenic types when known, are listed in Table I. All cultures were incubated at 37°.

TABLE I.—*Characters of Strains Used.*

Strain.	Lactose.	Maltose.	Xylose.	Antigenic type.	Amino acid sensitivity.
145	+	+	+	U	Serine, norleucine.
75	−	−	+	U	,, ,,
E2380 (V)	+	+	+	0111 B4 H12	,,
E2846	+	+	+	0111 B4 H12	,,
E.C.	+	+	+	U	,, ,,
21	+	+	+	—	Norleucine, lysine.
E2070	+	+	+	U	,,
A170	+	+	+	0111 B4	,, serine, cystine.
A104	+	+	+	0111 B4	Norleucine, serine.
E1790	+	+	−	U	,, ,,
E1662	+	+	+	0111 B4 H2	,, norvaline.
E3751	+	+	+	0111 B4 H12	,,
E044	+	+	−	044	,,
A172	+	+	+	0111 B4	,, ,, serine, cystine.
147	+	+	+	—	Norleucine, norvaline, cystine.
146	+	+	+	—	Norleucine.
F2206 (AV)	+	+	+	0111 B4 H12	,,
K12	+	+	+	—	,, valine, norvaline.
E2186	+	+	+	0111 B4 H2	Serine, cystine.
197	+	+	+	—	Norleucine, norvaline, lysine.

U = unknown antigenic structure.
All strains are indole +, methyl red +, Vosges-Proskauer −, citrate −, mannitol +.

Test animals.—Most of the work was done using a pure strain of Swiss white mice No. 1, weight limits 18–22 g. For one experiment albino guinea-pigs, weight 300 g. were used.

Virulence tests.

The method used was similar to that described by Smith (1950) and consisted of intraperitoneal injection into mice of 0·2 ml. of a suspension of the organism (14 hr. growth in 10 per cent mouse serum broth) in 2 per cent gum tragacanth or in 1·7 per cent hog gastric mucin (1701 W. Wilson Labs., Chicago) + 0·4 per cent finely powdered charcoal. The gum tragacanth had the effect of increasing the virulence of all the strains about 50-fold, thereby extending the scale over which differences in virulence could be observed. The gastric mucin + charcoal was even more effective in this direction and results were more reproducible. Without virulence-enhancing factors, some of the less virulent organisms would not kill even when 0·2 ml. of an undiluted 14-hr. culture was injected, whereas with the most virulent strains a 1/100 dilution without mucin would kill. For many of the strains 3 10-fold dilutions of the suspensions were injected into 3 groups of 3 mice, *i.e.*, 9 mice in all. This rough method was supplemented with some of the strains by additional determinations using 6 mice in each group.

The results with the gum tragacanth suspensions were all obtained on the same day with one batch of mice; all the organisms had been inoculated in equal numbers into tubes prepared from the same batch of 10 per cent mouse serum broth.

Viable counts on the original bacterial suspension were done in triplicate by the drop method of Miles and Misra (1938). Deaths usually occurred within 24 hr. of injection but final results were read after 48 hr. In the initial stages of the work, heart blood cultures were performed on all dead mice, but since these cultures were always positive for the specific *Bact. coli* injected, the laborious process of isolation and identification was later discontinued.

Toxicity tests.

The organisms were grown for 14 hr. in 10 per cent serum broth, centrifuged and concentrated in saline. The thick suspensions were heated for 30 min. at 80° and samples withdrawn for sterility tests and for dry weight determination after allowing for the Na Cl content. In order to be comparable with the virulence tests, bacterial suspensions were mixed with hog mucin and charcoal in such proportion that the mixture contained 5–30 mg. bacterial dry wt./ml. and 1·7 per cent w/v hog gastric mucin + 0·4 per cent w/v powdered charcoal.

Each suspension was then injected intraperitoneally in 0·2 ml. amounts into 4 mice.

TABLE II.—*Virulence and Toxicity of* Bact. coli *Strains for Mice.*

Strain.	LD_{50} 14 hr. culture + 1·7% hog mucin. organisms.	LD_{50} 14 hr. culture + 2% gum trag. organisms.	Lethal dose killed organisms in mg./dry wt.†	Serine sensitivity. μg./ml.
*145	10^3	10^5	4·4	2
* 75	10^3	10^5	2·3–5	4
*E2380 (V)	4×10^3	—	5·0	—
E2846	2×10^4	—	3·0	500
E.C.	2×10^4	—	2	—
21	4×10^4	10^6	2·5–4·5	30
E2070	10^5	10^5	—	15
A170	5×10^6	10^7	4	15
A104	at least 10^7	10^6	—	4
*E1790	„ 10^7	10^7	4	8
E1662	„ 10^7	2×10^7	—	30
E3751	„ 10^7	—	—	500
E044	„ 10^7	10^8	2·1	250
A172	„ 10^7	—	3·5	15
147	„ 10^7	3×10^7	3·5	8
146	„ 10^7	10^8	3·0	15
*E2206 (AV)	„ 10^7	—	6·0	500
*K12	„ 10^7	—	—	250
E2186	„ 10^7	10^6	2·8	15
197	„ 10^7	4×10^8	2·5	2

* The average of many tests performed on different occasions.
† Figures given killed 1–3 out of the 4 mice used.

Calculation of LD_{50}.

Approximate LD_{50} values were calculated by the method of Reed and Muench (1938). While the small numbers of mice make most of the data very approximate, the differences in virulence between the most and least virulent strains were far greater than the experimental errors. In particular, with some of the strains such as 75, E1790, E2380 and E2206 (see Table II) on which further work has been based, the virulence tests have been repeated many times and the LD_{50} values are those derived from at least 100 mice in each case. For the sake of simplicity strain E2380, which is a typical virulent strain, is referred to as V and E2206, which has the same antigenic structure but is nevertheless avirulent, as strain AV.

RESULTS.

Virulence determinations, using such small numbers of mice for each test, are obviously inaccurate and results from repeat tests sometimes differed by a factor of 5. Nevertheless, the results leave no doubt that there are very large differences in mouse virulence among these strains. The most virulent need only 10^3 organisms to kill, whereas some others do not kill when more than 10^7 organisms are injected.

It is clear from Table II that there is no connection between serine sensitivity and virulence for mice. The results of the toxicity tests are likewise crude, but show that the lethal doses of killed organisms lie within similar limits for the many different strains. This would seem to rule out the production of a heat-stable toxin by the virulent strains only, and since all the strains have simple growth requirements we must look elsewhere for explanations of these great differences in killing power.

Three virulent and 3 avirulent strains were chosen for further work. From Table III it can be seen that strains AV (E2206) and V (E2380) differ greatly in virulence yet possess the same antigenic structure, the same sugar fermentations and are inhibited by the same amino acids. It seems unlikely that mouse virulence is directly connected with any of these properties. This was confirmed for sugar fermentations and amino acid inhibitions by developing mutants changed in various characters and testing the mutants for virulence. Table III shows that in no case was the virulence altered.

TABLE III.—*Characteristics of* Bact. coli *Strains and Mutants.*

Strain.	Lactose.	Maltose.	Xylose.	Antigenic type.	Amino acid sensitivity.	LD_{50} organisms.
75	−	−	+	U	Serine, norleucine	10^3
75 serine-resist.	−	−	+	U	Norleucine	10^3
75 lac. +	+	−	+	U	Serine, norleucine	5×10^3
V15	+	+	−	U	„ „	5×10^6
K12	+	+	+	U	Valine, norleucine	2×10^7
K12 mucoid	+	+	+	U	„ „	10^7
K12 valine resist.	+	+	+	U	Norleucine	2×10^7
E2206 (AV)	+	+	+	0111 B4 H12	Serine, norleucine	2×10^7
E2380 (V)	+	+	+	0111 B4 H12	„ „	4×10^3

U = Unknown antigenic structure.

Tests for heat-labile extra-cellular factors.

To see if the virulence of some strains might be due to the elaboration of an extra-cellular aggressin or heat-labile toxin, after the fashion of the *Clostridia* (Evans, 1945), 14-hr. cultures of strains 75 and E1790 were centrifuged and the supernatants sterilised by filtration through a gradocol membrane. The sterile

supernatant from E1790 was added to the cells of 75 and vice versa and the organisms re-suspended. Dilutions were made from these and simultaneously of cells from 75 and E1790 which had been washed 3 times in saline. Virulence tests made with these suspensions showed no variations from 75 whether washed, suspended in its own supernatant or in that from E1790. Similarly with E1790 cells. We are left with the conclusion that in these cases virulence is not mediated by some powerful extra-cellular property.

Effect of injecting killed cells together with living ones.

In order to see if virulent cells, when killed, produced a virulence-enhancing effect on injection of avirulent cells, dilutions of *Bact. coli* AV were made so as to straddle the LD_{50} dose and injected together with 10^7 killed, washed *Bact. coli* V. No enhancement of killing was found over the results to be expected from the injection of strain AV alone. This is contrary to the results of a similar experiment by Maaløe (1948) using avirulent strains of *Salmonella typhi murium*.

Growth curves in vivo.

Experiments *in vitro* had failed to show any differences in growth rate between the strains when growing in 10 per cent mouse serum broth. Attempts were made to study the growth of these organisms *in vivo* by the simple method of washing out the peritoneum at intervals following injection. Model experiments showed that it is possible to recover 50–80 per cent of an injected dose of organisms by sacrificing the mouse, skinning it from tail forwards, opening the peritoneum and washing out with 10 ml. of sterile saline.

Groups of 20 mice were inoculated with the same dose of *Bact. coli* + mucin and charcoal. Two of the mice were sacrificed immediately and bacterial counts done on peritoneal washings. At the same time counts of the dose injected were made. At intervals during the following 16 hr., pairs of mice were killed and counts were made of the numbers of intraperitoneal organisms. Fig. 1 shows the results of such an *in vivo* growth curve using 75 and E1790 as the test organisms. From this it can be seen that there is little difference in growth rate between the two strains in the logarithmic part of the curve. The difference is in the capacity to continue multiplying from a small inoculum ; when 8000 organisms of E1790 are introduced they multiply for a few hours and are then slowly eliminated.

Presumably these *in vivo* growth curves are the resultant of two opposing tendencies ; these are on the one hand the multiplication of the organisms and on the other their removal or inactivation by host defence mechanisms. The difference between the virulent and avirulent organisms in this case resolves itself into a difference in behaviour towards one or other of these defence mechanisms.

Peritoneal leucocytosis.

Examination of stained peritoneal smears taken from mice at varying intervals following infection with 10^7 organisms of *Bact. coli* V or AV without mucin, showed that with both organisms polymorphonuclear leucocytes began to appear in considerable numbers after about 8 hr. Large numbers of organisms were phagocytosed, but there was no consistent difference between the strains in the

average numbers per leucocyte. The presence of mucin in the peritoneum did not affect the numbers of leucocytes migrating into the cavity to any marked degree, but the extent of phagocytosis was reduced with both virulent and avirulent organisms.

Injection of *Bact. coli* cytoplasm prepared by mechanical disruption of a bacterial suspension with ballotini beads (Cooper, Rowley and Dawson, 1949)

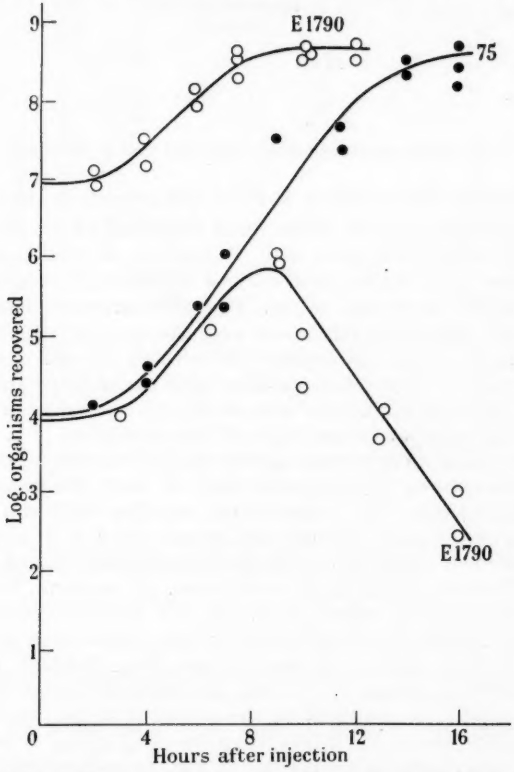

FIG. 1.—Growth of *Bact. coli* strains E1790 and 75 in the mouse peritoneum. 0 = numbers of organisms of E1790 recovered in the total peritoneal washings at varying times following I.P. injection of 8×10^3 and 10^7 organisms. ● = same after I.P. injection of 10^4 organisms of strain 75.

showed that under these conditions both organisms attract similar numbers of leucocytes. In one such experiment injection of 0·4 mg. dry weight of cytoplasm from *Bact. coli* V or AV into each of 2 groups of 10 mice produced after 18 hr. an average of 10^6 leucocytes (mostly polymorphs) per peritoneum irrespective of whether V or AV cytoplasm had been used.

That leucocytes played an indecisive rôle in defending against this artificial infection, in the presence of mucin, is shown by an experiment in which peritoneal leucocytosis was produced in mice by injection of *Bact. coli* AV cytoplasm as above. The mice were challenged 18 hr. later by inoculation with varying doses

of organism V + mucin and charcoal. As a result the survival time of the mice was increased but the final mortality figures were the same as in a normal control group (Table IV).

TABLE IV.—*Survival of Mice when Challenged Intraperitoneally after Induction of Leucocytosis.*

Numbers of *Bact. coli* V injected.	Leucocytosed mice dead at		Normal control mice dead at
	24 hr.	48 hr.	24 hr.
10^5	3/4	4/4*	4/4
2×10^4	2/4	4/4*	4/4
2×10^3	0	3/4*	4/4

* The numbers include those which had died at 24 hours.

Effect of Mouse Blood on Bact. coli *Strains* in vitro.

Mice were anaesthetised with ether, bled aseptically from the heart and the blood defibrinated with a bent glass rod. Quantities of 0·5 –1 ml. of this blood were pipetted into bijou bottles and various dilutions of organisms AV and V were added, contained in 0·1 ml. saline. The dilutions were designed to cover a range of 50–50,000 organisms/ml. blood and the bottles rotated in a machine kept at 37° (Robertson and Sia, 1924). Samples of 0·1 ml. were withdrawn at intervals during 4 hr. and spread on nutrient agar plates for counting. In all such experiments no bactericidal action was observed and after a few hours the organisms began to multiply irrespective of their virulence.

A similar test using defibrinated guinea-pig or human blood from several normal individuals showed in every case that at least 500 organisms/ml. blood were killed within 30 min. This observation together with the knowledge that one of the differences between human and mouse blood is the lack of detectable haemolytic complement in the latter, tempted speculation that this might account for the failure of normal mouse blood to kill *Bact. coli in vitro*. Table V shows the results of an experiment in which *Bact. coli* AV and V were rotated in bijou bottles containing mouse blood and fresh or heat-inactivated guinea-pig serum, and human blood as a control. It can be seen from Table V that addition of 0·4 ml. fresh guinea pig serum to 0·7 ml. defibrinated mouse blood produces a system which is rapidly bactericidal to the avirulent strain, but has little or no effect on the virulent strain V. When the guinea-pig serum is heated to destroy complement, all bactericidal power is lost. In Fig. 2 the data from several survival curves of this type are collected and show the striking difference in behaviour of the two strains.

Similar differences in survival rates, reflecting their differences in virulence, were found between strains 145 and 75, and E1790 and E3751.

When tested in guinea-pigs for virulence in the same manner as for mice, strain 75 had an LD_{50} of 5×10^4 and strain E1790 of 10^8 organisms. The inter-strain difference in virulence is of the same order in guinea-pigs as in mice, suggesting that the same defence mechanisms are involved. In the guinea-pig, normal serum which contains complement is actively bactericidal *in vitro*, and when diluted suitably will differentiate between virulent and avirulent strains. Although the relationship between mouse virulence and resistance to the bactericidal effects of mouse blood and complement-containing sera have been shown,

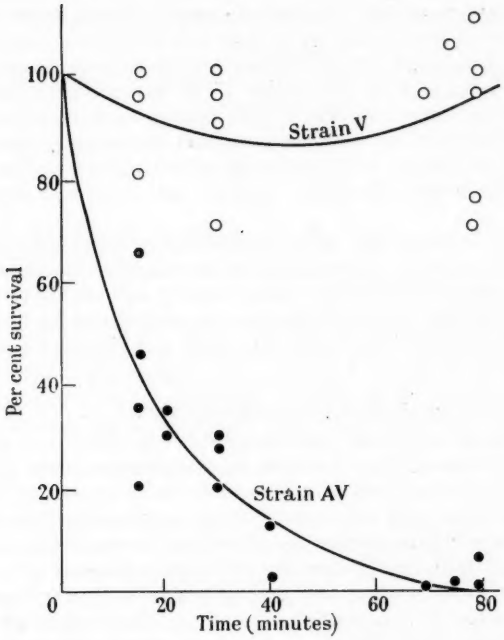

FIG. 2.—Survival of *Bact. coli* strains V and AV in mouse blood and guinea-pig serum. 0 = strain V. ● = strain AV. Experimental details as in Table V.

TABLE V.—*Survival of* Bact. coli *Strains AV and V* in vitro.

Time mins.	Human blood.		Mouse blood + heated serum.		Mouse blood + unheated serum.	
	AV.	V.	AV.	V.	AV.	V.
5	22	50	75	65	84	56
20	0	0	40	60	52	46
35	0	0	66	64	12	33
75	0	0	> 100	> 100	6	65

To 0·7 ml. defibrinated mouse blood, 0·4 ml. sterile guinea-pig serum (active or inactivated by heating) was added, followed by 700 AV or 600 V in 0·1 ml. saline. Figures are numbers of colonies obtained by plating out 0·1 ml. of the mixture.

this does not conclusively prove that this is the mechanism by which the mouse itself differentiates between virulent and avirulent strains, since there is no bactericidal action by normal defibrinated mouse blood, nor does this possess any haemolytic complement.

Bactericidal effect of sera.

Earlier experiments on the survival of strains 75 and E1790 in normal mouse serum + 10 per cent guinea-pig serum had failed to show any killing. This was now found to be due to the anticomplementary action of mouse serum, each ml. of mouse serum being able to neutralise 25–30 haemolytic units of complement, and since guinea-pig serum contains approximately 80 units of complement, about 50 per cent of guinea-pig serum must be added to mouse serum to give any free complement. Mouse serum with sufficient added guinea-pig serum to give free complement differentiates between virulent and avirulent strains like mouse blood + serum.

It is doubtful whether the anticomplementary property develops in mouse serum during the processes of obtaining it, or whether it exists *in vivo*. In any case the test for mouse complement, using a sheep cell rabbit antiserum indicator, is by no means infallible, and it is possible as pointed out by Cushing (1945) that complement activity might be found by using a different indicator system.

Bactericidal power of mouse blood following infection.

If the differences in mouse virulence of these *Bact. coli* strains are to be accounted for in terms of their reaction to complement, then either complement must be present in mouse blood *in vivo* or it must appear in the course of the infection. Complement may only occur locally, as suggested by Maaløe (1948), or it may be generalised throughout the blood and tissue fluids of the animal. It may be significant that the *in vivo* growth curves in mice (Fig. 1) showed that discrimination between virulent and avirulent strains by the host did not appear until some hours after the start of the infection. This suggested that the bactericidal factor, whether it be complement or not, only appears in the mouse as a result of the stimulus of infection.

To test this suggestion, 30 mice were inoculated with 10^7 AV without mucin. Two mice were sacrificed every second day and their serum titrated for haemolytic complement activity and for anticomplementary activity to guinea-pig serum. On the same day 3 more mice were bled and the defibrinated blood tested for bactericidal power towards *Bact. coli* AV. No trace of complement appeared and the anticomplementary power of the mouse serum stayed constant at 30 antihaemolytic units/ml. On the other hand, by the 12th day the defibrinated mouse blood had marked bactericidal powers (Table VI). This Table also shows that the different behaviour of strains AV and V is quantitative rather than qualitative. Thus, although normal mouse blood + guinea-pig serum does not kill strain V, convalescent mouse blood with the same amount of added guinea-pig serum is markedly bactericidal to strain V, although not so rapidly as to strain AV. Any effect on the counts due to clumping of the bacteria should be the same with both strains. This protection by convalescent blood is also found *in vivo* since immunisation of mice with either strain V or AV will protect the mice against a subsequent challenge 12 days later with over 100 lethal doses of *Bact. coli* V. It seems likely that both these effects are due to the common antigenic structures of these two strains and that the increased bactericidal activity of convalescent blood is a function of increased antibody content.

In spite of the failure to detect haemolytic complement in mouse blood the evidence strongly suggests that a small amount of complement must be present

TABLE VI.—*Survival of* Bact. coli *V and AV in Normal and Convalescent Defibrinated Mouse Blood, with and without added Guinea-pig Serum.*

Time mins.	Normal mouse blood 0·7 ml. + guinea-pig serum 0·4 ml.		Convalescent mouse blood 1 ml.		Convalescent mouse blood 0·7 ml. + guinea-pig serum 0·4 ml.	
	AV.	V.	AV.	V.	AV.	V.
Start	78	48	61	58	75	68
15 mins.	78	61	42	44	11	29
30 mins.	18	49	22	34	0	5
75 mins.	2	57	1	23	0	4

Figures are numbers of colonies obtained by plating 0·1 ml. samples. Initial counts were 70 colonies AV/0·1 ml. and 60 V/0·1 ml.

Convalescent mouse blood obtained from mice injected 12 days previously with 10^7 AV.

in convalescent mouse blood and that this is responsible for its bactericidal effect. This evidence was obtained by demonstrating that treatment of convalescent mouse serum or guinea-pig serum with zymosan, which is known to inactivate the third component of complement (Bier, Leyton, Mayer and Heidelberger, 1945), simultaneously removes all bactericidal properties from the sera.

DISCUSSION.

It has been found that among *Bact. coli* strains there are a few which possess very much greater virulence for mice and guinea-pigs than the majority. In the first analysis the difference between these two groups is reflected by the capacity of the virulent strains to grow up and multiply from a small inoculum ; in other words, the growth rate of the virulent bacteria *in vivo* must exceed the overall rate of destruction by the body's defences.

Since the use of mucin in this system increases virulence about 500 times and most strains of *Bact. coli* are relatively avirulent without mucin, then mucin must reverse one of the effective body defences, probably phagocytosis. This may be analogous to the case of streptococci, reported by Hadley and Wetzel (1943), where there was a great increase in mouse virulence in passing from the rough to the smooth encapsulated form, this increased virulence being presumably due to resistance to phagocytosis. A further even larger increase in virulence was obtained by continued animal passage, without changing in any apparent way the state of dissociation or antigenic properties of the organism. The similarities to the present findings with *Bact. coli* are that addition of mucin increases virulence of all strains, possibly by providing them with an artificial capsule, and enabling them to resist phagocytosis ; over and above this effect however, there are great differences in virulence which appear to be related to resistance to some unstable serum factor, probably complement.

If there are several distinct host defence mechanisms, it is reasonable to suppose that against any given microbial species some of the mechanisms will play a predominant rôle over the others. If the majority of the strains of such a species are avirulent then the occasional virulent strain will presumably be one which is resistant to the one or more normally dominating defence reactions. In the event of the difference in virulence between two strains being due to a different behaviour towards *one* defence reaction, then the magnitude of the difference in virulence will be a measure of the predominance of that particular defensive

reaction over all the others. Conversely, where there are great changes in magnitude between the virulence of two strains of the same organism it seems likely that there is a key defence reaction among the several available, which is involved in the struggle against that organism. The present model infection of mice with *Bact. coli* strains provides such a case since there are a few strains which are about 10,000-fold more virulent than the majority. This great increase in virulence is associated with resistance to the bactericidal effect of normal mouse blood plus complement-containing sera, as has been described by Maaløe for *Salm. typhi murium* (1948). It is suggested that resistance or otherwise to this effect is the major determinant of mouse virulence in *Bact. coli*.

SUMMARY.

Twenty strains of *Bact. coli* have been tested for mouse virulence by intraperitoneal injection together with 1·75 per cent hog gastric mucin. Six of these strains were virulent in comparison to the remainder. This virulence is not due to the possession of a heat-stable toxin since all the strains have approximately the same toxicity when killed.

In vivo studies show that virulence is a reflection of the capacity to continue multiplying in the peritoneum from a small inoculum. This capacity for sustained growth *in vivo* is correlated with greater resistance towards the *in vitro* bactericidal powers of complement-containing sera. It is suggested that this resistance to complement is an important determinant of mouse virulence in *Bact. coli*.

The excellent technical assistance of C. R. Jenkin is gratefully acknowledged.

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THE EFFECT OF CORTISONE ON SECOND SET SKIN HOMOGRAFTS IN RABBITS.

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Received for publication July 26, 1954.

EARLIER papers (Billingham, Krohn and Medawar, 1951*a*, *b*) which showed that cortisone can prolong the survival time of a first set of skin homografts also showed that neither systemic nor local treatment with cortisone can significantly modify the response to a second set of homografts in a rabbit that has been "immunized" as a result of an earlier grafting operation with skin from the same donor. Locally applied cortisone was also unable to protect one set of grafts from the consequences of a concomitantly developed immunity stimulated by another set of homografts (from the same donor), which was not being treated locally with cortisone. It was concluded that the main effect of cortisone was to retard the development of systemic immunity and not to interfere with the expression of that immunity at the site of the grafts.

Cortisone was not administered in any of these earlier experiments until the day of the second operation. It is, therefore, reasonable to suppose that the grafts were destroyed by pre-existing circulating antibody* before cortisone could have any effect on the tissues which are responsible for the production of antibodies. Since the animals had only received the first set of grafts about two or three weeks previously their immunity to the donor skin was probably at its maximum.

Fischel, Stoerk and Bjørneboe (1951) studied the rate of disappearance of passively administered antibody in cortisone-treated rabbits and concluded that cortisone does not influence the rate of breakdown or excretion of existing antibodies, but that it inhibits the synthesis of fresh antibody proteins. It seemed possible, therefore, that, if treatment with cortisone was begun some time before the second operation, the continued formation of fresh antibody to skin homografts might be prevented in an animal which had already received one set of grafts. During the time necessary to achieve this end the antibodies already existing when treatment began would gradually be broken down in the normal way. If skin from the original donor were then grafted a second time to such a cortisone-treated animal the grafts would not be immediately affected by

* The word antibody is used in this paper as a convenient term to apply to the substances responsible for the systemic immunity that develops in a recipient animal after it has received skin homotransplants. It is recognized that actual antibodies have not yet been isolated from the plasma of such "immune" animals. Billingham and Sparrow (1954) have, however, provided evidence for the existence of a protective iso-antibody in the serum. They have shown that dissociated Malpighian skin cells, which had been treated *in vitro* for 22 hr. with excess serum from a rabbit that had been specifically "immunized" against them, have lost their normal ability to proliferate when transplanted to an area of granulations.

the attack of a pre-existing immunity, but might react more like first set grafts. If treatment with cortisone were continued their survival time might also be prolonged, just as is the survival time of first set grafts.

MATERIALS AND METHODS.

Mature male and female rabbits of different breeds were used. Full thickness pinch grafts (9 mm. in diameter) from the dorsum of the ear of the donor (chosen from another breed) were transplanted on to an open raw area prepared on the side of the chest, the general technique being similar to that described by Billingham and Medawar (1951). The first step in each experiment was to transplant 8 homografts of skin and to allow them to break down. None of the grafts was removed for histological examination, but naked-eye inspection showed that all the grafts had broken down by the 9th day after operation. The animal was then presumed to have become immunized. The second grafting operation, at which another set of 8 homografts from the original donor was transplanted to a raw area prepared on the opposite side of the chest, was carried out 37, 41, 44, 50 and 60 days after the first. Treatment with cortisone was begun at varying intervals before the second operation and was continued until the end of the experiment. One rabbit in each group of animals comprising a single experiment received no cortisone and was used as a control. The other members of each group received preoperative treatment with cortisone (usually 10 mg. cortisone acetate daily by subcutaneous injection) for 1, 11, 12, 14, 15, 21 and 25 days. The second set of grafts was first inspected 6 days after the operation and thereafter at 3-day intervals. Biopsy specimens were removed at the time of each inspection in order to assess the survival time of the grafts histologically.

In one experiment a set of grafts derived from a new donor was also grafted at the time of the second operation so that the behaviour of first and second set grafts could be studied side by side. Two sets of grafts from different donors (one a first set, the other a second set) were also used for two further experiments in which treatment with cortisone was not begun until the day of the second operation. Details of the experiments are given in the Table.

At the end of each experiment the animals were killed and the liver and adrenals dissected out and weighed.

RESULTS.

Behaviour of second set grafts in untreated animals.

The general behaviour of these grafts differed in no way from that described previously by Medawar (1945). The biopsy specimens removed 6 days after operation showed a thin indolent epithelium on the point of destruction. The grafts were not swollen; vascularisation was inadequate and the circulation stagnant. Round cell infiltration was only slight and those cells which were to be seen in the dermis were usually dead. Breakdown time was always estimated at 7 days or less. Rabbit 130 received the first immunizing set of grafts 108 days before the second set—the longest interval elapsing between the two operations in this series of experiments. The histological appearance of the biopsy specimens from this rabbit suggests that the immune reaction did not develop so rapidly nor so intensely as in the other animals, whose first operation had been carried out more recently. The response resembled much more closely the normal acute inflammatory reaction to a first set of grafts.

Behaviour of second set grafts in animals treated with cortisone.

The period of pre-treatment with cortisone enabled the second grafts to survive for a longer period of time and more convincingly than when treatment began only at the time of the second operation. The epidermis of many of the grafts

TABLE.—*The Effect on Second Set Skin Homografts of Treatment with Cortisone begun before the Second Operation.*

No. of rabbit and sex.	Interval between 1st and 2nd operation (days).	Duration of pre-treatment with cortisone (days).	Daily dose (mg.).	Duration of treatment after grafting (days).	Body weight at start of experiment (g.).	Weight loss during experiment (g.).	Weight of liver (g.).	Weight of both adrenals (mg.).	Area of wound on 12th post-operative day (per cent of original).	Breakdown time (days)	
										1st set homo-grafts.	2nd set homo-grafts.
142.F.	41	21	10	13	2540	455	90	285	72	—	>13
146.F.		11	10	26	3390	610	111	151	81	—	>23
145.F.		Control	—	13	2530	160	84	535	54	—	7
138.M.	44	21	7·5*	13	2000	+155	106	242	93	—	13
136.M.		12	3·75	13	1700	230	86	587	83	—	7
137.M.		12	7·5	13	1920	165	96	269	80	—	8
139.M.		Control	—	13	1660	90	38	389	60	—	7
156.M.	50	25	10	13	3000	0	157	438	—	—	>13
153.F.		25	10	13	3225	652	210	141	54	—	10
154.M.		15	10	13	2875	+175	256	267	81	—	10
157.M.		15	10	13	3050	250	214	296	61	—	9
152.M.		Control	—	13	2950	150	65	507	42	—	7
88.F.	60	22	10	15	2750	770	"large"	132	74	—	14
89.F.		12	10	16	2350	310	200	92	90	—	13
90.F.		1	10	15	3050	+100	238	275	77	—	>15
91.F.		Control	—	15	2750	90	93	336	39	—	6
105.M.	37	14	10	15	2200	200	116	174	69	>15	7
106.M.		Control	—	15	2100	100	44	266	48	7	<6
131.F.	78	0	10	15	2550	—	187	258	52	>15	10
132.F.		Control	—	9	3750	—	81	481	73 (9th day)	7	7
129.F.	108	0	10	15	3600	—	192	296	66	>15	10
130.F.		Control	—	8	2400	—	88	370	62 (8th day)	8	7

* For the first 4 days this animal received 10 mg. daily.

removed 6 days post-operatively was proliferating, a result which is never seen in normal second set grafts. The extent of the improvement in survival time was not always very impressive, nor did the grafts usually achieve the prolonged life that is characteristic of first set grafts in rabbits which have been given cortisone (Billingham, Krohn and Medawar, 1951*a*).

The three experiments in which two sets of grafts, one from the original and one from a new donor, were grafted at the time of the second operation show clearly how specific the response to homografts is. There seems to have been no significant antigenic overlap between donors. The histological appearance of the two types of graft was quite different and entirely appropriate to the sort of graft, whether first or second set. Thus, the fact that one group of grafts was being destroyed by a pre-existing immunity did not modify the typical first set reaction to the other grafts. This was especially true in the rabbit whose second operation was performed only 37 days after the first. Cortisone treatment enabled the new first set grafts to survive in the usual way despite the immediate and severe reaction to the other set, which indicated the presence of specific antibodies in the circulation.

The development of granulation tissue.

During the experiments an incidental observation was made on the circumstances in which the granulation tissue of the prepared raw area may develop while a rabbit is being given cortisone. Usually the fine vessels which run in the panniculus are readily visible 12 or 15 days after the wound has been made, and there is practically no growth of granulations in the wound area during treatment except exactly under the grafts. Outgrowth of epithelium from the circumference of the graft is very slight or absent. If, however, epithelium manages to grow out at all, a thick pad of granulation tissue develops immediately beneath the epithelium to the extent of the outgrowth but no further. There is no way of deciding, at present, whether the epithelium grows out only as far as granulations permit, or whether granulations only develop where they are covered or stimulated by the graft or its outgrowing epithelium.

Weight of liver and adrenal glands.

As was expected, the liver increased and the adrenals decreased in weight during the period of treatment. The changes were not appreciably greater than those which follow a shorter period of treatment, nor did the rabbits which received the largest doses of cortisone for the longest time necessarily show the greatest alteration in the weights of the organs. The general processes of repair, as judged by the extent of the contraction of the wound 12 days after operation, seem, however, to have been further impeded by the pre-treatment with cortisone.

DISCUSSION.

These experiments show that the usual reaction to a second set of skin homografts can be modified by treating the recipient rabbit with cortisone for about 25 days before the second operation. Treatment for this length of time does not, however, completely restore the conditions prevailing before the rabbit had received a first set of grafts, for, although the life of the second set is often pro-

longed and the response modified in the direction of a first set response, such grafts never survive for as long as do first set grafts treated with cortisone. The most reasonable explanation for these results seems to be that the treatment interfered with the synthesis of new antibody protein, but that the duration of pre-treatment with cortisone was insufficient or the dose inadequate to prevent completely the synthesis of antibody globulin. It may also be that a period of 10–25 days does not allow sufficient time for the complete removal of any antibody protein that existed at the start of the injections. The half life of passively administered γ-globulin in human beings seems to be only 3–4 days, but experiments in which isotopic methods were used have suggested that the half life of actively formed globulin is about 2 weeks. The work of Dixon, Buckantz, Dammin and Talmage (1953) indicates that antibody protein disappears more rapidly in rabbits. They find that homologous γ-globulin and passively transferred homologous antibody both have a half life of 4–5 days only. On this basis it might be thought that a period of 25 days should have been long enough almost to clear antibody from the circulation. But the experimental conditions in which the antibody protein was administered in the work mentioned above may be sufficiently unnatural for an unphysiological result to be obtained, nor may the rate of disappearance of homologous γ-globulin necessarily give a true picture of the rate at which natural antibody disappears. Since the antibody to skin homografts has not yet been identified, its concentration in the plasma cannot be measured, and consequently there is no direct way of deciding its rate of destruction. Nevertheless these experiments justify the belief that treatment with cortisone, if persisted in, can inhibit even the immune response to a second set of skin homografts.

Billingham, Brent and Medawar (1954) have reported that active immunity weakens perceptibly in mice after about four months. The single experiment in which the second set of grafts was not transplanted until just over three months after the first operation suggests that the rate of decrease in active immunity in rabbits is of the same order.

SUMMARY.

Treatment with cortisone is known to prolong survival of skin homografts transplanted from one rabbit to another (first set grafts). A second series of skin grafts from the original donor of the first set (second set grafts) is more rapidly destroyed than the first set and the process of destruction presents a different histological picture. Treatment with cortisone, beginning on the day of the second operation, does not significantly modify this response.

If treatment with cortisone (10 mg. daily) is started 25 days before the second operation, the intensity of the usual immunity response is decreased and the grafts behave more like first set grafts. Survival time is increased.

It is suggested that this treatment inhibits the formation of fresh " antibody " and allows time for the excretion or metabolism of " antibody " circulating before treatment began.

The state of heightened resistance to skin homografts from a given donor is declining 108 days after the first grafting operation.

The suspension of cortisone acetate (Merck) was supplied by the Medical Research Council.

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THE HEALING OF LOCALISED AREAS OF NECROSIS IN THE COLON OF THE MOUSE.

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Received for publication July 26, 1954.

THERE does not appear to be any comprehensive account of the amount of regeneration to be expected following experimental damage to the mucous membrane of the colon. The membrane is made up of closely-set tubular glands and the normal histological appearance makes it clear that cell division occurs continuously in the bases of these glands, so that there is a continuous replacement of the cells of the superficial portions of the glands and of the intervening surface epithelium. Such a method of cell renewal, it may be assumed, is comparable with that demonstrated in the small intestine by Leblond and Stevens (1948) and in the stomach by Stevens and Leblond (1953). Thus it might be expected, if the damage to the colonic mucosa were superficial and the bases of the mucous glands were left intact, that the mechanism which normally effects cell replacement could restore the damaged mucosa to normal. In the stomach Grant (1945) has shown that superficial damage to the gastric mucosa is repaired in this way. However, if the whole thickness of the mucous membrane including the bases of the mucous glands is destroyed, regeneration in this way could not be expected, since it would be necessary for the denuded area to become covered by migration of cells derived from the adjacent mucous membrane.

Complete destruction of localised areas of the mucous membrane of the colon has been produced in the mouse by the application of silver nitrate, and the healing of the lesions studied.

MATERIALS AND METHODS.

The animals used were laboratory stock white mice of no particular strain. The results recorded are those obtained from well grown animals weighing 25–35 g.; both sexes were used, but the results were not significantly different.

The mice were anaesthetised with ether and the colon exposed by an abdominal incision in the lower midline. Through the anus was inserted a thin-walled glass tube about 3 cm. in length and 2–3 mm. internal diameter. By way of the abdominal incision the inserted end of this tube was identified within the lumen of the colon about midway between the anus and the liver. A second glass tube was inserted within the first which it fitted nicely. The inserted end of this second tube was filled with fused silver nitrate crystals, which formed a mass protruding slightly beyond the end. The tube was manipulated so that the silver nitrate was pressed against the mucous membrane from within the lumen of the bowel and an area of necrosis produced on the ventral aspect of the colon. The size of this area could be determined by superficial inspection through the abdominal wound. It was approximately circular in shape and involved about one-third of the circumference of the bowel. It was not possible by external inspection to determine the depth of the lesion, so that it was only by histological examination that it could be certainly decided whether the mucous membrane had been destroyed in its whole thickness or whether the damage was superficial, leaving intact the bases of the mucous glands. After the necrosis had been produced the tubes were

withdrawn and the abdominal wound sutured. About 50 per cent of the animals survived the operation and the lesions were sectioned at intervals subsequently. Bouin's solution was used as a fixative and the sections were stained with haematoxylin and eosin.

RESULTS.

Although the depth of the lesion could not be determined by inspection at the time of operation, complete destruction of the mucosa could be assumed if, when subsequently examined, sections of the lesions showed destruction of the muscle layers. In such lesions the breach in the bowel wall was filled by granulation tissue and adhesions. Thirty-five such lesions were examined at varying periods up to 35 days after infliction. All lesions had in common the absence of any sort of epithelial covering of the main and central part of the lesions, and a typical example of this common finding is seen in Fig. 4.

In lesions examined immediately after operation it was seen that they were not of uniform depth. In the centre all layers were destroyed, but at the periphery the lesions became more superficial and there was a peripheral area in which the bases of the mucous glands were left intact (Fig. 1). It is thus necessary to consider separately the healing of this peripheral area in which destruction of the mucosa was partial, and that of the central portion in which it was complete and the muscular layers destroyed.

Following the incomplete necrosis in the peripheral area there was a rapid reaction in the mucous membrane. Within 24 hours the necrotic portion of the mucosa was seen to be separating and new surface epithelium had formed between the remaining portions of the mucous glands (Fig. 2). When the fate of these glands was followed in sections made at later stages examples were found in which they did not regain their normal height (Fig. 4). Furthermore, there were instances of branched glands as shown in Fig. 5. Such glands are found in the normal mucous membrane, but so rarely that their presence may be ascribed to the injury inflicted.

Other results were relevant to the degree of regeneration to be expected following partial destruction of the mucosa. These were obtained from experiments in which it was a reasonable deduction that destruction of the mucosa was only partial over the whole area of the lesion. When these lesions were examined the muscle layers were found to be intact. although they had undergone some fibrosis ; the site of the lesion was identified by the presence of external adhesions. An example of such a lesion, 28 days after operation, is shown in Fig. 8. In this case, as in all others, external examination at the time of operation showed that some necrosis of the bowel wall had been produced. This could not have involved the muscularis mucosae because this layer is present in Fig. 8, and McMinn and Mitchell (1954) have shown that in the ileum of the cat the muscularis mucosae has no regenerative capacity. Since it cannot be considered likely that over the whole lesion the necrosis stopped precisely between the bases of the mucous glands and the muscularis mucosae, it must be concluded that in parts, at least, of the lesion the bases of the glands remained intact. From Fig. 8 it will be seen that the mucous membrane at the site of the lesion does not differ significantly in histological appearance from the normal.

In lesions where complete destruction of the mucous membrane was indicated by the absence of the muscle layers, there was, over the main part, no evidence

of new formation of epithelial cells (Fig. 4). However, immediately adjacent to the persisting mucous membrane some new cell formation was observed. This consisted of an outgrowth of an epithelial layer, usually one cell thick, which did not extend for more than 500μ over the granulation tissue. It showed no tendency to increase with time and dividing cells were not seen in it. In all the lesions examined there was no evidence of new gland formation in this epithelial layer.

To this description, applicable to all lesions, may be added, as amplification, descriptions of single lesions. The outgrowth of the epithelium began within 4 days and Fig. 3 shows that it can take place while the lesion is still filled with exudate and necrotic tissue. In another lesion examined 9 days after operation (Fig. 5) this epithelial outgrowth extended over the surface of the lesion for about 500μ which was the maximum distance observed. In this case the epithelium was closely applied to the underlying granulation tissue and showed two small depressions. These cannot, however, be put forward as evidence of new gland formation because in adjacent sections they are no deeper than seen in Fig. 5; moreover this was the only instance of such depressions occurring in the 35 specimens examined. That the spread of the epithelium was no more extensive in lesions examined subsequently is shown in a lesion sectioned after 16 days (Fig. 6). In this Figure the derivation of the epithelium from persisting mucous glands is demonstrated.

In examining the lesions in which destruction of the mucosa was complete the subjective impression was gained that the area of the lesion decreased as time went on. It is therefore relevant to cite a case in which, after 27 days, the lesion had become reduced to a transverse slit. In a longitudinal section the site of the lesion was indicated by adhesions (Fig. 7) and the absence of the muscle layers showed that the mucosa had been completely destroyed. Although the area was covered with mucosa it will be seen that there are two edges which are in contact but which have not united. Further, both these edges resemble those of other lesions of equivalent age by showing no evidence of new gland formation. The appearance in Fig. 8 is therefore attributed to the approximation of the mucosal edges by the contraction of the underlying tissues.

DISCUSSION.

It seems probable that if only the superficial part of the mucous membrane were destoyed, cell division in the remaining bases of the mucous glands would lead to repair of the damage. Following the production of a lesion deduced to be of such an extent the expectation is realised by the restoration of the histological integrity of the damaged mucous membrane (Fig. 8). However, if the area of mucous membrane that is superficially damaged is adjacent to an area in which all layers of the bowel wall are destroyed, complete restoration of the mucous membrane may not follow (Fig. 4). It is therefore necessary to qualify to this extent the conclusion that, in the present experiments, recovery followed partial destruction of the colonic mucous membrane.

If the whole thickness of the bowel wall had been destroyed, and the breach had become filled with granulation tissue and adhesions, the only evidence of regeneration of the mucous membrane was an outgrowth of epithelium over the granulation tissue. This outgrowth was confined to the periphery of the damaged area, most of which remained uncovered by epithelium. No new gland formation

was observed in the outgrowth. Because of the limitations of the technique used it was not possible to determine directly whether this comparative failure of regeneration would have occurred had the mucle layers not been destroyed. However, it is to be expected that even had these remained after destruction of the mucosa the damaged area would have undergone an inflammatory reaction with the production of granulation tissue, as McMinn and Mitchell (1954) found in the ileum of the cat. Thus, whether the muscle layers were destroyed or not, regeneration would take place on the surface of granulation tissue and might be expected to follow a similar course.

These findings may be compared with those of other authors who removed or destroyed localised areas of the alimentary mucosa, leaving intact the main part of the muscle layers. In the duodenum Florey and Harding (1935) found that resection of the mucous membrane in the cat was followed by the regeneration of glands resembling those of the pyloric region of the stomach. In addition Brunner's glands and the villi were re-formed. McMinn and Mitchell (1954) performed similar experiments on the ileum of the cat and observed the complete re-formation of normal mucous membrane. In the stomach the regenerative capacity of the mucosa after its complete loss has been found to vary. The re-formation of mucous crypts, but not chief and parietal cells, was found in rats, guinea-pigs and rabbits by Wynn Williams (1953), who destroyed areas of mucosa by the application of heat. On the other hand, Ferguson (1928), who resected areas of mucosa, could find no evidence of regeneration in the rabbit; in the dog, however, he found that all elements of the gastric mucosa were re-formed. In general these results indicate that the mucosa in other regions of the alimentary canal has a higher regenerative capacity than the mouse colon, in which regeneration is confined to outgrowth of an epithelial layer in which new gland formation is not observed.

The variability of the results obtained by Wynn Williams (1953) and Ferguson (1928) after damage to the gastric mucosa raises the possibility that the regenerative capacity of the colonic mucosa in different animals may vary similarly. For that reason it is only with reservations that the results of the present experiments

EXPLANATION OF PLATES.

FIG. 1.—Immediately after operation. The necrosed tissue is demarcated from the surviving by a line in which silver salts have been deposited. At the periphery of the lesion the bases of the mucous glands are undamaged; centrally all layers have undergone necrosis. (×60.)

FIG. 2.—Nineteen hours after operation. The necrosed tissue is separating. There is a new formation of epithelium over the surviving mucous membrane. (×90.)

FIG. 3.—Four days after operation. There is a commencing epithelial outgrowth into exudate and necrotic tissue. (×95.)

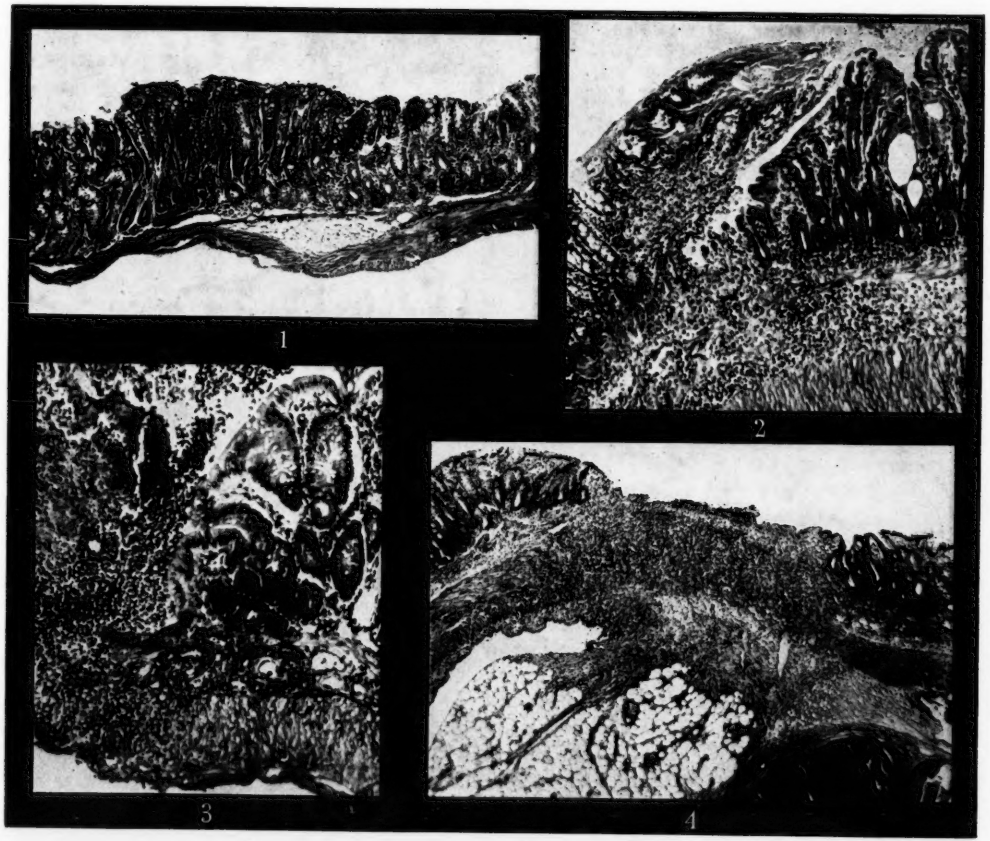
FIG. 4.—Nine days after operation. The mucosa and muscle layers have been destroyed and the breach is filled with adhesions and granulation tissue. There is no epithelial covering of the greater part of the lesion. On the left-hand side of the lesion the mucous glands are of less than normal height. (×40.)

FIG. 5.—Nine days after operation (an enlargement from Fig. 4). There is an epithelial outgrowth from the remaining mucous membrane. Note the branched mucous gland (×100.)

FIG. 6.—Sixteen days after operation. The epithelial outgrowth is seen arising from a persisting mucous gland. (×105.)

FIG. 7.—Twenty-seven days after operation. The site of the lesion is indicated by adhesions. The muscle layers are destroyed. The edges of the mucous membrane have come together but have not united. (×25.)

FIG. 8.—Twenty-eight days after operation. The site of the lesion is indicated by an adhesion. The muscle layers are intact but have undergone some fibrosis. The mucous membrane shows no significant abnormality. (×40.)



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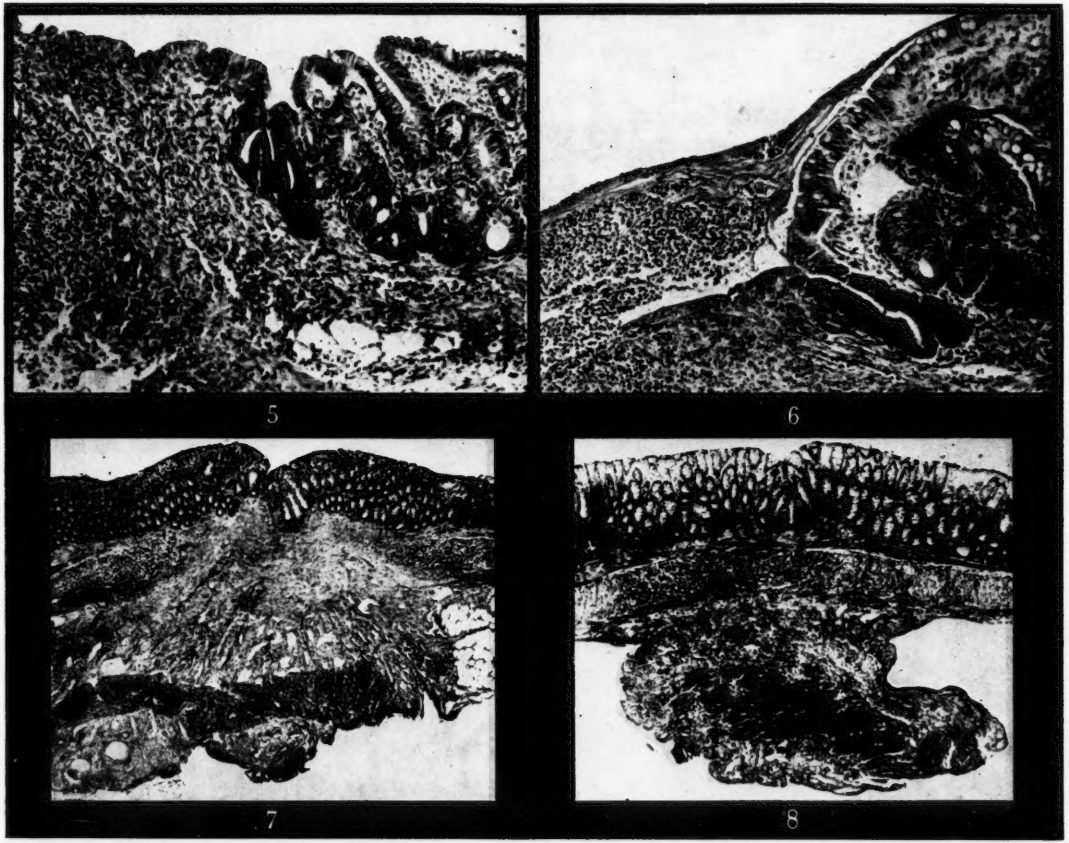

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are discussed in relation to the healing capacity of the human colon. Suggestions have been made that there are conditions in which the loss of areas of human colonic mucosa is followed by its re-formation (Felsen, 1945). It may be that, in these conditions, certain other possibilities have not been excluded. The first is that the loss was not complete and the bases of the mucous glands remained, so that without new gland formation the mucosa might regain its normal appearance, as in the experiment illustrated by Fig. 8. Another is that contraction of the muscle coats and of fibrous tissue might bring the edges of the lesion together ; Fig. 7 illustrates this process. Thirdly, it is possible that the regeneration described in the human bowel might be confined to the covering of the denuded area by a layer of epithelium in which, as in the mouse, there was no new gland formation. Dukes (1954) has observed this in a study of ulcerative colitis. He states that if the ulceration reaches the submucosa or the muscle layers the denuded area becomes covered with an epithelial layer in which new gland formation is very rare.

Illustrations of the lesions of ulcerative colitis (Dukes, 1954 ; Brooke, 1953) suggest that this epithelial layer can become sufficiently extensive to cover areas of some size. It may be assumed that, as in the mouse, the cells of the outgrowing layer originate from persisting mucous glands and that the maintenance of the integrity of the layer would depend on the continuous renewal of its cells by division and migration of cells in these glands. The more extensive the epithelial layer became the more difficult would be its renewal and the more precarious its integrity. It might therefore break down under conditions of trivial strain and constitute a factor in recurrent ulceration of the colon.

SUMMARY.

Localised areas of necrosis have been produced in the wall of the mouse colon by the application of silver nitrate.

If both the mucous membrane and the muscle layers were destroyed the breach in the wall of the colon became filled with granulation tissue and adhesions. In such areas regeneration of the mucosa was confined to the formation of an epithelial layer partially covering the damaged area. No new formation of mucous glands was seen in this layer.

At the periphery of these lesions the necrosis of the mucous membrane was superficial, leaving intact the bases of the mucous glands. Recovery from this partial lesion did not always follow, but when the bases of the mucous glands were left intact over the whole of the damaged area the normal histological appearance was regained.

There are limitations to the application of these results to other animals, but they have a possible bearing on ulcerative conditions of the human colon.

These experiments were performed while receiving a personal grant from the British Empire Cancer Campaign. It is a pleasure to acknowledge the technical assistance of Miss E. R. Blake.

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MORPHOLOGY OF THE ENLARGED LIVER IN GUINEA-PIGS TREATED WITH ACTH OR CORTISONE.

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Received for publication July 27, 1954.

DURING investigations into the relationship between vitamin C and adrenal function, the unexpected observation was made that the continuous administration of ACTH or cortisone to guinea-pigs was followed by a massive increase in the weights of their livers; this was seen also in rabbits, but not in rats or several other species examined (Harris, Bland, Hughes and Constable, 1953). This observation has since been confirmed in experiments on guinea-pigs at the National Institute of Health, Bethesda, U.S.A. (Reid, 1953, personal communication).

The present work was undertaken to find what morphological changes accompanied this enlargement of the liver. Chemical analyses had previously shown that the enlargement was characterized by an increase in the content of water and glycogen in the liver together with a gradual addition of protein (Harris *et al.*, 1953). Histological examination of the livers was made to determine whether the increase in weight was due to a true growth by cellular multiplication or to the enlargement of existing liver cells. For this analysis the mitotic rate of the liver cells and the percentage of binucleate cells were estimated, and measurements of individual liver cells were made. If the increase in weight were due to true growth a high mitotic rate would be expected, and possibly also an increase in the percentage of binucleate cells which are formed by a complete nuclear division without cell cleavage. As already briefly indicated in the earlier paper (Harris *et al.*, 1953), the main conclusion reached has been that the increased weight goes parallel with, and is proportional to, an increase in the size of the liver cells, which in turn is attributable to enlargement of the cytoplasm.

MATERIALS AND METHODS.

In these experiments, the feeding and dosing of the animals were done at the Dunn Nutritional Laboratory. Young guinea-pigs weighing 300–350 g. were given a course of intramuscular injections of ACTH (2 mg. Armour standard LA-I-A, four times daily for 2–10 days) or cortisone acetate (3 injections of 5 mg. each day for 10 days). In the experiments with ACTH the controls were given injections of normal saline or left uninjected; the control animals in the cortisone experiment were injected with the "aqueous vehicle" used for suspending the cortisone. The guinea-pigs in the ACTH experiment (test and control) were fed on a natural mixed diet of bran, oats, roots, etc., and those in the cortisone experiment on a scorbutogenic diet supplemented with ascorbic acid (Harris *et al.*, 1953). The rats used were injected intramuscularly with 5 mg. per day of cortisone acetate, and were kept on the standard synthetic diet in use at the Dunn Nutritional Laboratory.

For histological examination the material was fixed in "Susa" solution, Carnoy's solution or formol-saline. The tissues fixed in "Susa" solution were stained with haematoxylin

and eosin, and those fixed in Carnoy's solution were treated with the periodic-acid Schiff reagent for glycogen, and were controlled by digestion for 1 hr. at 37° with saliva (Lillie, 1948). Frozen sections were cut from the tissues fixed in formol-saline, and were stained for fat with Sudan 3.

A quantitative histological analysis was made of the livers of 10 guinea-pigs treated with ACTH and of 14 control animals, and also of 5 guinea-pigs treated with cortisone and of 5 corresponding controls. The pancreas and adrenals were examined in 10 guinea-pigs treated with ACTH and in 14 controls. In the experiments on rats treated with cortisone, the livers of 14 treated and of 14 control animals were examined histologically. The sections used for the quantitative analysis were cut at 10 μ.

The mitotic rate and the percentage of binucleate elements were determined by counting the number of mitotic and binucleate cells respectively per 500 liver cells. This was done in the livers of guinea-pigs treated with ACTH and with cortisone, and in the controls of both groups. In addition, in each section, 100 liver cells were measured with the aid of a micrometer eyepiece. The cell measurements were made in two diameters at right angles to each other, and the half of the mean of the two was taken as the value indicating the radius of the cell. The estimate of the mean cell volume was based on the measured mean radius, the cells being assumed to be spherical.

RESULTS.

Effect of ACTH on the Liver of Guinea-pigs.

Livers from 10 treated and 14 control animals were examined histologically. The standard course of injections of ACTH was given, as mentioned above ; even after 2 days a marked increase in the liver weights was noted, as compared with the controls. This increase continued throughout the course of the injections, and chemical analyses show that it was due to extra glycogen and water, and later to an increase in protein.

The histological analysis showed that in both the treated animals and the controls the mitotic rate varied from 1–2 per cent, and the percentage of binucleate cells from 2–5 per cent (Table I). From the measurements of the liver cells, it was found that in all the animals there was some variation in the size of the liver cells, but that in the treated guinea-pigs there were more large cells than in the controls. Table II gives the mean radius with standard deviations of the liver cells for the control and the ACTH-treated animals after varying periods of injection ; the difference in the mean radius betweeen the control and treated animals is also shown. It will be seen that, with the exception of one pair of guinea-pigs treated for 2 days, the mean radius of the liver cells was always significantly greater in the treated animal than in the corresponding control. The average of the mean radius of the cells in the control animals was 7·57 μ (range 7·07–7·87 μ), and in the ACTH-treated animal it was 8·38 μ (range 7·75–8·73 μ). The estimated average of the mean volume of the cells in the treated animals was 2481·5 cu.μ, and in the controls 1837·5 cu.μ. The average percentage increase in cell volume in the treated animals was thus 36 per cent (S.D. $\pm$ 2·96), and the corresponding figure for the percentage increase in liver weight was 37 per cent ($\pm$ 9·9) when expressed as a percentage of body weight and 36 per cent ($\pm$ 11·7) when uncorrected for increase in body weight—a remarkably close agreement.

It was necessary to ascertain whether the increase in cell volume was accompanied by an enlargement of the nuclei. The nuclei were measured by the same method as that used for the entire cells. It was found that the mean diameter of the nuclei in both the controls and the treated animals varied from 6–7 μ.

TABLE I.—*Effect of ACTH on Mitotic Rate and Number of Binucleate Cells.*

Days of injection	Per cent of Mitoses. ACTH.	Mitoses. Control	Per cent of Binucleate cells. ACTH.	Binucleate cells. Control.
2	2	2	2	5
	1	2	3	3
4	2	1	2	2
	1	1	2	4
6	1	1	3	2
	2	1	4	2
8	1	2	2	2
	2	1	5	3
10	1	2	2	3
	2	1	4	4

TABLE II.—*Effect of ACTH on Size of Liver Cells.*

Days of injection	Mean radius of liver cells (μ). Control.	ACTH.	Increase.
2	7·44 ± 0·116	8·51 ± 0·124	1·07 ± 0·172
2	7·82 ± 0·119	8·0 ± 0·093	0·18 ± 0·151
4	7·38 ± 0·113	8·30 ± 0·114	0·92 ± 0·162
4	7·45 ± 0·101	8·53 ± 0·116	1·08 ± 0·154
6	7·63 ± 0·101	8·37 ± 0·118	0·74 ± 0·155
6	7·70 ± 0·120	8·72 ± 0·137	1·02 ± 0·18
8	7·07 ± 0·091	7·75 ± 0·107	0·68 ± 0·138
8	7·70 ± 0·108	8·42 ± 0·127	0·72 ± 0·165
10	7·65 ± 0·109	8·73 ± 0·134	1·08 ± 0·176
10	7·87 ± 0·110	8·50 ± 0·118	0·63 ± 0·162

The livers of the guinea-pigs treated with ACTH contained very much more glycogen than those of the controls, as shown by histological analysis with the periodic-acid Schiff reagent, and this agrees with the macro-chemical results (see also Harris *et al.*, 1953). All the sections stained for glycogen were controlled by digestion with saliva. In the majority of the treated guinea-pigs the glycogen was evenly distributed throughout the liver lobules, but in some animals it was concentrated around the central vein. The frozen sections stained with Sudan 3 showed that the liver cells contained little stainable fat, and histologically there was no gross difference in fat content between the liver cells of the controls and those of the treated animals.

Effect of Cortisone on the Liver of Guinea-pigs.

Livers from 5 guinea-pigs given 15 mg. cortisone acetate daily for 10 days, and from 5 controls given injections of normal saline for 10 days, were examined histologically. As with ACTH, there was a great increase in liver weight and also in the content of water, glycogen and protein after cortisone treatment. The histological findings also were similiar to those in the ACTH-treated guinea-pigs. The mitotic rate varied from 1–2 per cent, and the percentage of binucleate cells from 2–4 per cent in both treated animals and controls, showing that the increase in weight could not be accounted for by new growth of liver tissue. The

same degree of variation in the size of the liver cells was found, as previously noted, in all the treated and control guinea-pigs examined, but in the treated animals there were more large cells than in the controls. The average of the mean radius of the cells in the control animals was 8·02 μ (range 7·82–8·16 μ), and in the cortisone-treated guinea-pigs 9·50 μ (range 9·13–9·83 μ). The greater mean radius in controls in this experiment than in the ACTH controls is attributable to the difference in diet already mentioned. The estimated average of the mean volume of the cells in the cortisone-treated animals was 3589·5 cu.μ and in the controls it was 2159·7 cu.μ; this represents a mean increase in cell volume of 66 per cent ($\pm$ 15·0) in the treated animals. The corresponding figure for the percentage increase in liver weight expressed as a percentage of body weight was 57 per cent ($\pm$ 15·7) and 51 per cent ($\pm$ 15·8) when uncorrected for increase in body weight—this, again, is in good agreement with the figure for the increase in cell volume.

Effect of Cortisone on the Liver of Rats.

Histological examination of the livers was made on 14 rats that had been treated with 5 mg. cortisone acetate daily for periods of 10, 20 or 40 days, and on 14 control animals injected with normal saline for the same periods. There was no significant effect on the weight of the liver or on the chemical composition of its cells after cortisone treatment, and in keeping with this no difference histologically in the size of the liver cells or in their glycogen content could be detected when the controls and treated animals were compared. The reason for this species difference is not yet known.

Effect of ACTH on Other Organs.

The pancreas and adrenals were examined histologically in the guinea-pigs treated with ACTH. In the pancreas, the islets of Langerhans varied appreciably in size in both treated animals and controls, and no morphological difference could be detected between the two. In the adrenals, no gross structural abnormalities were noted, but no cell measurements were made.

DISCUSSION.

The massive increase in the weights of the livers of guinea-pigs after continued administration of ACTH or cortisone reported previously (Harris *et al.*, 1953) was shown to be accompanied by an increase in glycogen, water and protein, but its mechanism was not fully determined. In the present experiments it is shown that there is no increase in the mitotic rate or in the percentage of binucleate cells, a result which confirms that there is no new growth of liver tissue. Measurement of the liver cells has shown that there is a significant increase in their size after treatment with ACTH or with cortisone, which is due entirely to an increase in the amount of cytoplasm, there being no enlargement of the nuclei. The percentage increase in cell volume corresponds very closely with the percentage increase in relative liver weights. Thus, the figures for the ACTH-treated animals are 36 per cent increase in cell volume and 37 per cent in relative liver weight, and for the cortisone-treated animals 66 per cent and 57 per cent respectively. It seems, likely, therefore, that the enlargement of the liver cells can be attributed to an increased storage of water, glycogen and protein.

The reason for the greater increase in relative liver weight and liver cell volume in the cortisone-treated as compared with the ACTH-treated guinea-pigs is not known. The quantitative difference between the effect of injections of ACTH and of cortisone might best be explained by the supposition that less cortisone was produced by the stimulation of the adrenals *via* the ACTH than when cortisone itself was injected.

SUMMARY.

The morphological changes in the livers of guinea-pigs after continued administration of ACTH or cortisone have been studied.

The large increase in liver weight after such treatment was shown to be due to an increase in the volume of existing liver cells, attributable to enlargement of the cytoplasm, and not to a new growth of liver tissue.

After treatment with ACTH the percentage increase in relative liver weight was 37 and in cell volume was 36; in cortisone-treated guinea-pigs the figures were 57 and 66 respectively.

One of us (C. P. C.) has pleasure in acknowledging the constructive criticisms of the manuscript by Dr. Honor B. Fell, F.R.S. and Dr. A. Glücksmann, and her indebtedness to the British Empire Cancer Campaign for a grant.

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A MEASURABLE TUBERCULOUS LESION IN THE THIGH OF THE MOUSE.

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Received for publication July 28, 1954.

IN the search for anti-tubercular agents, the infection frequently used has been that produced in mice by the intravenous injection of tubercle bacilli (Martin, 1946). Despite its widespread use and the information gained from it, however, it is doubtful whether this type of infection and the conditions under which it responds to treatment are sufficiently close to those obtaining in man to make it the ideal experimental lesion for the assessment of chemotherapeutic agents intended for human use. Since the results are based on the times taken for treated and untreated mice to die, the infection must be overwhelming and, for convenience, capable of killing the untreated animals within three or four weeks. Because of the necessary severity of the infection, untreated animals rapidly lose weight after the first few days and become less resistant to adverse environmental influences. The intervention of intercurrent infection, for example, makes this test troublesome to laboratories which cannot carry large numbers of mice, but a more serious disadvantage is that the lowered resistance must apply to the tuberculous infection itself. Related to this suppression of resistance is the relentless progress of the disease, once established, in the face of adequate treatment with potent chemotherapeutic agents. The intravenous test appears, therefore, to give rise to an infection in which the natural defences of the host play only a minor rôle in determining the outcome of the infection and it is thus far from simulating the conditions commonly encountered in human tuberculosis.

On these considerations we thought it worth while to investigate the possibility of producing a measurable localised infection which was, if possible, ultimately self-limiting and which did not interfere with the general health of the animals. Such a tuberculous infection has been produced by a technique analogous to that of Selbie and Simon (1952) who showed that the virulence of staphylococci can be studied by their capacity to cause lesions in the thighs of mice after intramuscular inoculation. Preliminary experiments indicated that when tubercle bacilli are injected in this way into mice, lesions develop which respond, even after establishment, to streptomycin and isoniazid, but it has become apparent that some modification will be necessary before the lesion can be used directly as a critical test in chemotherapeutic studies. Moreover, thinking that some anomalies in the response to chemotherapy of experimental tuberculous lesions in general might be related to differences in behaviour between so-called primary and secondary tuberculosis, we also investigated the development of the lesion in animals which had been previously infected.

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In the present paper, we describe the production and development of the lesion and changes which occur when the same lesion is produced in these animals for a second time.

MATERIALS AND METHODS.

Cultures of the mouse-virulent human strain of *Mycobacterium tuberculosis* H37Rv, originally kindly supplied by Dr. P. D'Arcy Hart of the National Institute for Medical Research, were used throughout these experiments. The culture pellicle on Proskauer and Beck's medium was harvested at 19–23 days and transferred to a Griffith's tube and ground for about 30 sec. The growth was then suspended in Dubos' Tween medium without albumen and shaken with glass beads for 30 min. After allowing any remaining gross particles to settle out by gravity, the suspension was standardized to twice the opacity of Brown's Tube No. 10.

Albino market mice, 19–21 g. in weight, were used. They were housed in boxes of 5 and fed once daily on mouse cubes (Associated London Flour Millers). Mice of either sex were used at random, but a note of sex and of pregnancy, where it occurred, was kept.

The inoculum, consisting of 0·1 ml. of the standardized suspension of tubercle bacilli, was introduced from behind into the muscles of the thigh close to the femur (Fig. 1). Inoculations were made into the left thigh except when mice were re-inoculated, in which case they were given into the right thigh and also into the right thigh of the corresponding control mice.

An assessment of the size of the lesion was made by measuring, to the nearest 0·5 mm., the medio-lateral width of the affected thigh with sliding calipers and subtracting from this measurement the width of the normal thigh.

EXPERIMENTAL.

A total of 100 mice were inoculated in the left thigh with a 22-day culture. Of these, 20 were allowed to develop without interference, and successive groups of 20 were re-inoculated in the right thigh 6, 9, 20 and 27 days after the first inoculation. At each re-inoculation, the same bacterial suspension was used to inoculate 20 new mice in the right thigh in order to show the course of the disease produced by that inoculum in fresh animals. Of each group of 20, 10 were kept for measurement of the lesions and 10 were killed at intervals for macroscopic and histological examination of the affected thighs, the results of which will be reported in detail elsewhere.

Behaviour of the infection in untreated mice.

The general behaviour of the lesions in the original 10 mice that were kept untreated is shown in Curve C in Fig. 2–5. Other examples in untreated mice are shown in Curve D of Fig. 2–5, which show the courses of the infections in the new mice inoculated as controls to the re-inoculated mice. At 24 hr. there is already a definite swelling which does not alter appreciably in size until about the 10th or 15th day, when there commences a slow but regular increase in size to about the 40th day. At this stage the lesions begin to point and sinuses develop. There then follows a period of intermittent discharge from the lesions with a gradual but irregular fall in size ending in complete resolution from the 100th day onwards.

Apart from the encumbrance of the lesion itself, the affected limb appears to suffer no loss of function throughout the course of the infection. Once intermittent discharge from the lesions has ceased, excepting rare accidental joint involvement and occasional contractures, the limbs are left apparently normal. The mice remain in good condition and increase in weight normally. The overall mortality rate was only 10 per cent during the 120 days of observation.

Macroscopic appearance of lesions.

The lesion is almost always found in a potential intermuscular space bounded by the semimembranosus and biceps femoris muscles (Fig. 1), but may occur on the medial side of the thigh between the semimembranosus and adductor magnus

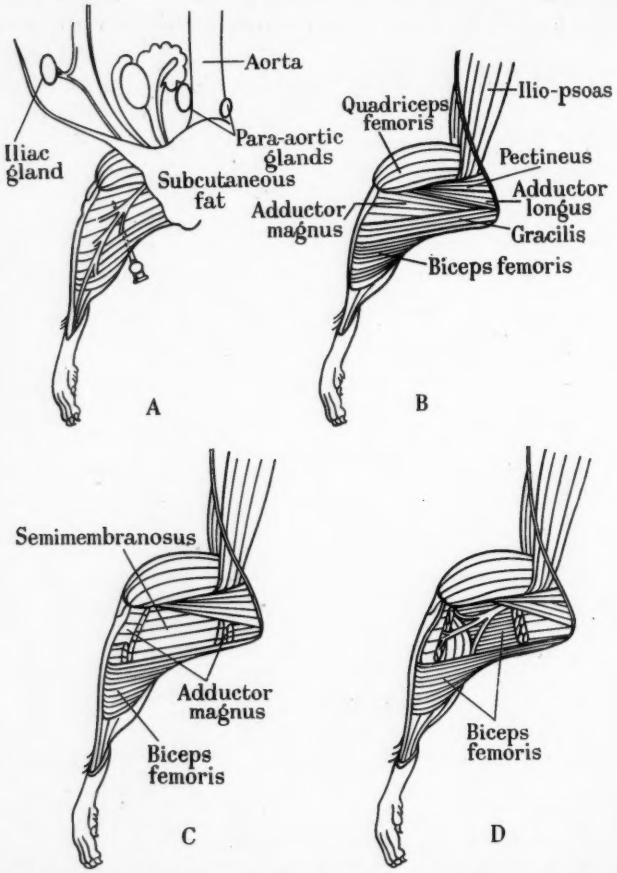

FIG. 1.—A. Superficial dissection of the thigh of the mouse, showing the site of inoculation. B. The adductor group of muscles which form the medial side of the thigh. C. The semimembranosus muscle which separates the medial and lateral intermuscular space. D. The lateral intermuscular space, between the semimembranosus and biceps femoris muscles, containing the sciatic nerve. It is in this space that the lesion usually develops.

muscles. During the first 3 or 4 days all that can be seen on dissection is the inoculum in the form of a flat disc about 2 mm. in diameter with some surrounding oedema. By the 5th day, however, a small abscess, or number of small abscesses, which later coalesce, can usually be seen. This lesion becomes definitely circumscribed by a thin wall at about the 10th day and then begins to increase in size.

The growing abscess now becomes compressed between the muscles, which are never seriously involved by the infection, and tracks usually anteriorly between the gracilis and biceps muscles, and occasionally posteriorly between the biceps and quadriceps muscles (Fig. 1). At these sites, the skin becomes adherent and a sinus or sinuses form, usually without infection of the subcutaneous space. When discharge has ceased and resolution is apparently complete all that remains

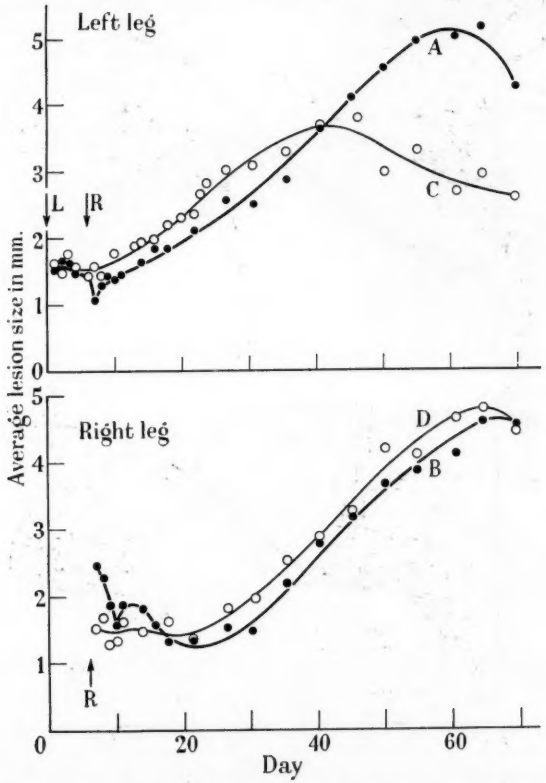

FIG. 2.—The effect of re-inoculation on the 6th day of the original infection. Curves A and B show the average course of the lesions in the left and right legs respectively of the group of animals re-inoculated on day 6. Curves C and D show the average course of the lesions in the two groups of fresh control animals inoculated on days 0 and 6 respectively.

is a small cleft in the muscle lined by a little silvery fibrosis. The discharges from these sinuses have been frequently examined bacteriologically, and no organisms, other than tubercle bacilli, have been demonstrated in them.

The regional lymph nodes show regular and early involvement, tubercle bacilli being present; the spleen becomes enlarged towards the end of the first week but tubercles are not found. No involvement of any other organ has been seen during or after the development of the lesions in these animals.

Effect of previous infection.

The general behaviour of the lesions produced by re-inoculation is shown by Curve B of Fig. 2–5, which compare the average course of the lesions in the groups of 10 re-inoculated mice kept for measurement with that of the lesions (Curve D) produced by the same inoculum in new mice. Twenty-four hours after inoculation the average size of the lesions in the previously infected mice is

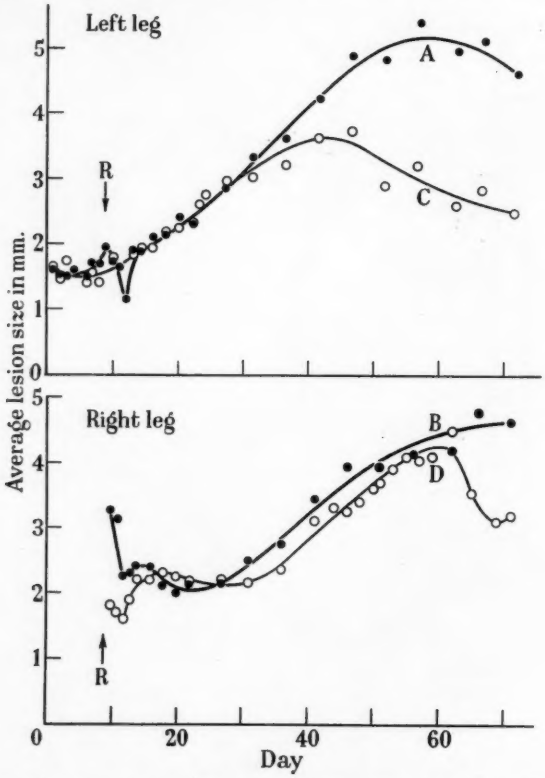

FIG. 3.—The effect of re-inoculation on the 9th day of the original infection. Curves A and B show the average course of the lesions in the left and right legs respectively of the group of animals re-inoculated on day 9. Curves C and D show the average course of the lesions in the two groups of fresh control animals inoculated on days 0 and 9 respectively.

about twice that found in the new mice; but within 3 days the lesions in the re-inoculated mice fall to about the same size as those in the new mice. The subsequent course of the second lesions varies with the length of the interval between the first and second inoculations. When this interval is 6 or 9 days the lesions follow the same course as that in the new mice (Fig. 2–3); but when it is increased to 20 or 27 days the second lesions in the re-inoculated animals increase in size definitely less rapidly and gain a smaller peak size than the lesions in the

control mice. These differences are more significant in the mice re-inoculated at 27 days than those re-inoculated at 20 days. In both cases, however, resolution starts at the same time and follows the same general course as in the control mice.

Effect of subsequent infection.

A striking change occurs in the existing lesion when these animals are re-inoculated (Curve A of Fig. 2–5). Twenty-four hours after re-inoculation the

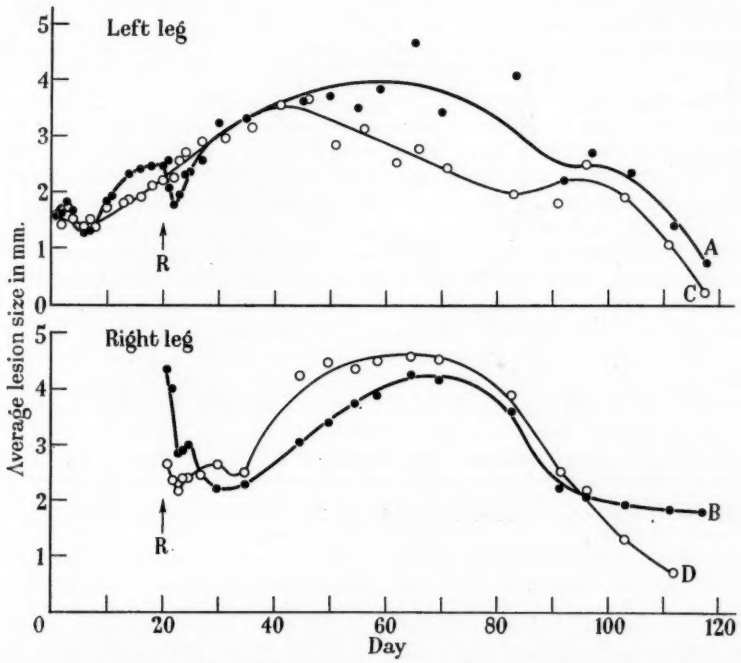

FIG. 4.—The effect of re-inoculation on the 20th day of the original infection. Curves A and B show the average course of the lesions in the left and right legs respectively of the group of animals re-inoculated on day 20. Curves C and D show the average course of the lesions in the two groups of fresh control animals inoculated on days 0 and 20 respectively.

first lesion is found to have fallen dramatically in size, and this reduction persists for a few days, the actual duration increasing with the interval between the first and second inoculations. Following this transient fall in size, the lesions return to their previous course and follow closely the rate of advancement of those in the controls. When the control lesions begin to form sinuses and enter their period of resolution, however, the lesions in the re-inoculated animals no longer follow, but continue to increase in size, reaching a greater peak value and resolving at a later period.

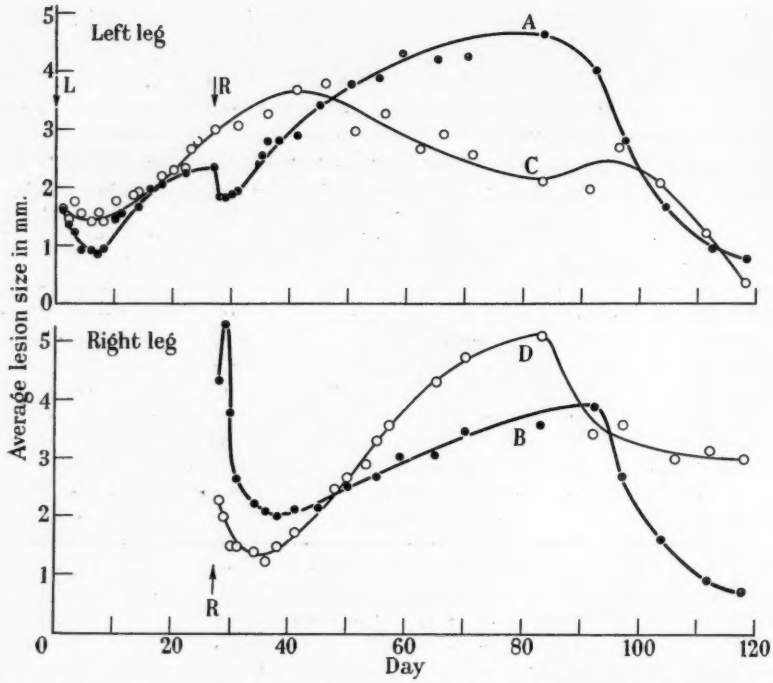

FIG. 5.—The effect of re-inoculation on the 27th day of the original infection. Curves A and B show the average course of the lesions in the left and right legs respectively of the group of animals re-inoculated on day 27. Curves C and D show the average course of the lesions in the two groups of fresh control animals inoculated on days 0 and 27 respectively.

DISCUSSION.

We have shown that the injection of tubercle bacilli into the thigh of the mouse produces an abscess which after a short latent period increases regularly in size until about the 40th day and then resolves by intermittent discharge through cutaneous sinuses, leaving the affected limb substantially normal. The general condition of the animals remains good throughout and there is no evidence of spread of the disease beyond involvement of the local lymph nodes and enlargement of the spleen.

Inoculation of the other thigh of mice previously infected in this way gives rise to an exaggerated response, maximal at 24–48 hr., having the characteristics of a hypersensitive reaction of the tuberculin type. Even at a time when hypersensitivity as manifest by this response is marked, however, skin hypersensitivity cannot be elicited in these animals either with tuberculin in any dilution or with heat-killed bacilli. This hypersensitive response is already seen in the animals re-inoculated at 6 days and is reproduced in each of the later groups. In the animals re-inoculated at 6 and 9 days, once the initial response is over, the lesions fall to the same size and follow the same course as those developing from the

same inoculum in fresh animals. It would therefore appear that hypersensitivity develops in these animals at a very early stage but that once the initial manifestations are over (2–3 days) the hypersensitive state is without any effect upon the subsequent development of the lesion. The presence of hypersensitivity is, in fact, associated neither with an immune type of response, nor with a more progressive or destructive lesion which Rich (1944) leads us to expect. In fact, the close agreement between the curves of development of the lesions in the hypersensitive and control animals suggests that the same controlling factors and the same systemic responses (and this must include hypersensitivity) are applied to each. Indeed, by the 6th day, hypersensitivity must have developed also in the control animals and unless the conditions in the primary infection are very remarkable, it is unlikely that a state capable of modifying the response of the body at large to tuberculous infection as profoundly as hypersensitivity does, is without effect on the original lesion. If the developing lesion is, in some way, isolated from the general hypersensitive state to which it gives rise, then it is apparent from the similarity of the lesions in fresh and previously infected animals that hypersensitivity, as represented by an exaggerated response to re-inoculation, has no effect on the course of the lesions. If, on the other hand, as seems more likely, developing tuberculosis gives rise to a general hypersensitive state such that the developing lesion itself implicates sensitive tissue, then the similarity in behaviour between the two sets of lesions must indicate that hypersensitivity is equally developed and active in both the freshly inoculated and re-inoculated animals. If, however, hypersensitivity is equally present, what is the meaning of the violent reaction in one group and the absence of any such reaction in the other? Without postulating anything of the mechanism, it may be suggested that the difference lies between organisms multiplying and spreading, in the course of a controlled infection, into tissues undergoing concurrent sensitisation, and a single mass of organisms injected into previously sensitised tissues. It is this kind of artificial elicitation of hypersensitive manifestations which has lead to the view that tuberculous infection exhibits enhanced destructivity in the presence of hypersensitivity. In the present experiments, injection of tubercle bacilli into pre-sensitised tissues leads to a violent response unquestionably associated with enhanced tissue destruction, but as far as the development of the continuing disease is concerned what is remarkable is not the virulence of the subsequent infection, but the rapidity of recovery from this severe " accident " and the striking absence of influence of any kind which it has on the final outcome of the disease. Although we do not know, at present, the importance of inoculum size on the course of these lesions, it seems equally fair to comment that in no case does the severe hypersensitive response appear seriously to have destroyed, altered or inhibited the tubercle bacilli of the re-inoculum.

With the re-inoculations of 20 and 27 days following the hypersensitive reaction, the lesions in the previously infected mice develop less rapidly and reach a smaller peak value than those in fresh animals receiving the same inoculum. Since the hypersensitive response is apparently unmodified, it appears that there has been added to the hypersensitivity seen in the early re-inoculations, a new reaction which has led to lesions which progress less rapidly and finally develop to smaller peak size, that is to say a reaction of immunity. This adds weight to the belief, at least for this lesion in this animal, that hypersensitivity and immunity are not identical, and that although hypersensitivity exists from a very early stage (Rich,

1944), immunity does not appear until about the third week (Dubos, Pierce and Schaeffer, 1953) after tuberculous infection. It is of interest to note that this immune state, while reducing the rate of progress and peak size of the lesion, does not accelerate the onset of resolution, or decrease the duration of the disease in any way. We have further shown that the course of the first lesion is profoundly modified by re-infection at another site. Concurrent with re-inoculation there is a temporary diminution in the size of the lesions followed ultimately by a more protracted evolution so that, while preserving the rate of progress of control lesions, they attain a greater maximum size and resolve later. The diminution of the lesions recalls the original experiments of Koch (1890) in which he observed that the ulcers and enlarged regional lymph nodes in tuberculous guinea-pigs were rapidly reduced in size by repeated injections of a highly diluted suspension of killed tubercle bacilli. It is, however, evident from our experiments that this type of procedure may ultimately lead to worsening of the disease. The mechanism of this relationship is not clear, but some clue may be given by the similarity between Curves A and C in Fig. 2–5.

It is apparent from these curves that in the re-inoculated animals the post-re-inoculation development of the first lesions corresponds closely, in each group, with that of the second lesions in the same animals, both in general shape and in time of resolution. This suggests that the later course of the disease is determined by the development of a new lesion arising at the time of transient reduction in size. In terms of actual magnitude, these lesions are larger at any given time than the corresponding secondary lesion, and it is likely therefore that they represent a combination of the original and induced lesions. This would mean that associated with the transient reduction in size of the first lesion is a temporary suspension, or marked diminution, in the defence response to the tubercle bacillus, so that organisms are liberated from the existing lesion and behave as a fresh inoculum, giving rise to a disease following essentially the same pattern as that due to the new inoculum in the second leg and superimposed on the continuing first lesion. That this combined lesion is subject to the same prevailing level of immunity as the second lesion in the same animal is shown by the similarity, in Fig. 6, of the flattened later part of Curve A and the course of the immune lesion represented by Curve C.

SUMMARY.

The production and development of a measurable tuberculous lesion in the thigh of the mouse is described. The lesion develops regularly for about 40 days and then resolves spontaneously by sinus formation, leaving the limb, after about 100 days, apparently normal. The animals suffer no apparent toxicity and there is no systemic spread of the disease beyond the regional lymph nodes.

Re-inoculation of the other thigh in animals already suffering from this lesion gives rise to an exaggerated response, maximal at 24–48 hr., and subsequently declining rapidly, having, that is, the characteristics of a hypersensitive reaction of the tuberculin type. This hypersensitive state is not associated with cutaneous hypersensitivity either to Old Tuberculin or to tubercle bacilli.

This hypersensitive response, which can be elicited as early as the 6th day of the disease, is without any effect on the subsequent course of the second lesion. The significance of this in terms of the possible part played by hypersensitivity in this tuberculous lesion is discussed.

Animals re-inoculated in the third week of the original infection exhibit, besides this hypersensitive response, an immune reaction leading to less rapid progression and reduced peak size of the second lesion as compared with controls. Despite this difference in behaviour the immune and control lesions enter their period of resolution at the same time and decline within the same period.

These findings support the contention that hypersensitivity and immunity are separate phenomena, that hypersensitivity exists from a very early stage, and that immunity appears, in the mouse, some time during the third week of infection. Immunity does not lead to an earlier or more rapid resolution of the lesion.

We wish to express our gratitude to Dr. P. D'Arcy Hart who kindly supplied the original strain of *Myco. tuberculosis* H37Rv, and to Mr. A. Honeycombe, Mr. D. Burnett and Mr. B. Allen for their patient and able technical assistance during the course of this work.

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CORTISONE AND THE METABOLIC RESPONSE TO INJURY.

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Received for publication August 9, 1954.

CERTAIN aspects of the metabolic response to injury, in particular the rise in the urinary excretion of nitrogen, sulphur, phosphorus and potassium and the considerable negative balance of these constituents following moderate to severe injury, have already been described by one of us (Cuthbertson, 1930, 1932, 1936, 1942). The most recent reviews (Engel, 1953; Selye and Horava, 1953; Cuthbertson, 1954) agree that the characteristic features of the metabolic reaction to physical injury are these negative balances and in the early stages impaired carbohydrate utilisation, fatty liver and tendency to ketosis. Sodium chloride and water retention occur particularly in the more serious cases, and there is normally an increase in blood and urine corticoids. The tissue changes are adrenal hypertrophy with depletion of ascorbic acid and cholesterol content, hypoplasia of lymphoid tissue and involution of the thymus, lymphopenia, eosinopenia and leucocytosis.

Since the work of Long, Katzin and Fry (1940), Ingle (1941) and Ingle and Thorne (1941) it has been known that the injection of adrenal cortical extracts or adrenal steroids containing oxygen substitution at the C_{11} position augments the urinary nitrogen excretion of fasting or normally feeding animals, and similar injections into adrenalectomized or hypophysectomized animals also raise the fasting nitrogen excretion above normal.

In the light of the work of Selye (1940), Ingle (1949), Kochakian and Robertson (1951, *a*, *b*), and our own observations, it was considered of interest to compare the effect on nitrogen metabolism of cortisone with that of an injury such as the fracture of a long bone. Eliel, Pearson and White (1952) had already shown that patients in whom hyperadrenocorticism occurred spontaneously or was induced by administration of adrenocorticotrophic hormone or cortisone exhibited losses of nitrogen, potassium and phosphorus strikingly like those of operative trauma.

It was decided to determine if injury and cortisone administration had similar effects on protein metabolism and what would happen if they were applied together, and to study this by reference to the urinary nitrogen excretion. A preliminary note on part of this work has already appeared (Campbell, Sharp, Boyne and Cuthbertson, 1953). It was also decided to examine the effect of adrenalectomy and to find whether cortisone possessed the so-called "permissive" or "conditioning" property as described by Ingle (1951) and Selye (1954) when fed to such adrenalectomized animals.

MATERIALS AND METHODS.

Male hooded rats of the Rowett Institute strain were used, 3 months old, average wt. 320 g., fed on the Institute's Stock Diet no. 86 of the following per cent composition : wheat 50, barley 25, white fish meal 7, meat and bone meal 6, dried brewer's yeast 5, dried grass meal 5, cod liver oil 1, sodium chloride 1.

They were housed in metabolic cages in a thermostatically controlled room (27°) and the urine and faeces were separated by Hopkins' arrangement. The flasks for the collection of urine contained 5 ml. 0·2 N-H_2SO_4 (1 ml. 5 per cent thymol in chloroform was added where samples were required for prolonged preservation). As it was known that appetite sometimes failed after operation in such experiments, the animals were first fed *ad lib.* 9.30–10.30 a.m. and 3.30–4.30 p.m. daily for 2 weeks. The appetite having then been gauged, they were given daily throughout the experiment a constant but somewhat smaller amount of food divided equally between these two 1-hr. periods.

During the feeding periods the cages were placed over filter paper and any urine passed was soaked out of the filter paper and added to the 24-hr. collection. The funnels were washed down twice daily, first with distilled water, and then with approximately 10 ml. 0·2 N-H_2SO_4. The combined urine and washings were filtered through glass wool, made up to a known volume, and an aliquot was taken for nitrogen estimation (Kjeldahl). When the urinary nitrogen output had steadied for 3 days the animals were subjected to treatment. The faeces were not collected, as previous work had shown that no change in the output of faecal nitrogen resulted from fracture (Cuthbertson, 1930, 1936 ; Howard, Parson, Eisenberg, Stein and Reidt, 1944) and it was also found that cortisone treatment caused no change. The treatments were the following :

Group I (Experiments 1–4).

Exp. 1.—(A) Cortisone acetate 25 mg. (B) Fracture of femur. (C) Cortisone acetate 50 mg. (2 tablets). (D) Fracture + cortisone acetate 25 mg.
1 rat was killed in group 1D before experiment was completed.

Exp. 2.—(A) 1 incision. (B) Dummy tablet. (C) Cortisone acetate 25 mg.

Exp. 3.—(A) 1 incision. (B) 2 incisions. (C) Cortisone acetate 50 mg. (1 + 1). (D) Cortisone acetate 75 mg. (2 + 1).

Exp. 4.—(A) 3 incisions. (B) Cortisone acetate 75 mg. (2 + 1). (C) Cortisone acetate 75 mg. (1 + 1 + 1). (D) Fracture + cortisone acetate 50 mg. (1 + 1).

Group II (Experiments 5–6).

Exp. 5.—(A) Sham operation followed after an interval by fracture. (B) Adrenalectomy followed after an interval by fracture.

Exp. 6.—(A) Sham operation followed by fracture ; cortisone acetate 0·8 mg./100 g. fed daily after sham operation. (C) Adrenalectomy followed by fracture ; cortisone acetate 0·8 mg./100 g. fed daily after adrenalectomy.

In each experiment in these two groups the animals were randomized by litters to treatment groups and they were killed 14 days after treatment. The size of the groups is shown in Table II.

Operative procedures.

All operations were performed under ether anaesthesia, which by itself was known to cause little, if any, disturbance of urinary nitrogen output. No sepsis occurred.

To induce fracture of a femur specially designed bone-breaking forceps were applied through a skin incision and after blunt separation of the muscle sheaths the bone was snapped at its midpoint as previously described by Cuthbertson, McGirr and Robertson (1939) and the wound stitched.

The cortisone tablets used were " Cortone " tablets (Merck) containing 25 mg. cortisone acetate and each weighed approximately 300 mg. For their implantation one, two or three incisions were made according to number. Where one incision was required it was made through the skin at the site on the outer aspect of the thigh corresponding to that used to induce fracture. By means of forceps the skin was separated from the underlying muscles

over an area large enough to hold one or two tablets as decided, which were then inserted and the wound stitched. Where two incisions were required comparable incisions were made on left and right thighs. For three incisions the procedure was as for two incisions, with a third made through the skin under the left shoulder.

As it was impossible to obtain dummy control tablets from the suppliers these had to be hand made. It was found by analysis that lactose formed the " vehicle " for the cortisone acetate tablets, and lactose dummy tablets containing 15, 20 and 25 mg. cholesterol were tested. The tablet eventually selected contained 15 mg. cholesterol and 285 mg. lactose and had the same weight as the "Cortone " tablets. It appeared to be " absorbed " at approximately the same rate.

The adrenals were removed through a dorsal incision. In the control animals the adrenal glands were exposed but not damaged. This constituted the sham operation.

Calculation of results.

The body weight of the animal was taken to be the average weight on the 5th, 4th and 3rd days before treatment. The basal nitrogen excretion was the average for the 3 days before treatment and was expressed as mg. N/100 g. body wt. The nitrogen excretion for 14 days following treatment was summed, expressed on the same basis, and 14 times the basal nitrogen was subtracted to give the additional nitrogen excretion as a result of treatment, again on a 100 g. body wt. basis. In animals which had undergone adrenalectomy or sham operation the body weight and basal nitrogen used to estimate the response to fracture were taken as the average obtained on the 3 days prior to fracture.

RESULTS.

The numbers used and the results are given in the Tables, and the Figure shows the effects in terms of magnitude and shape of response curves.

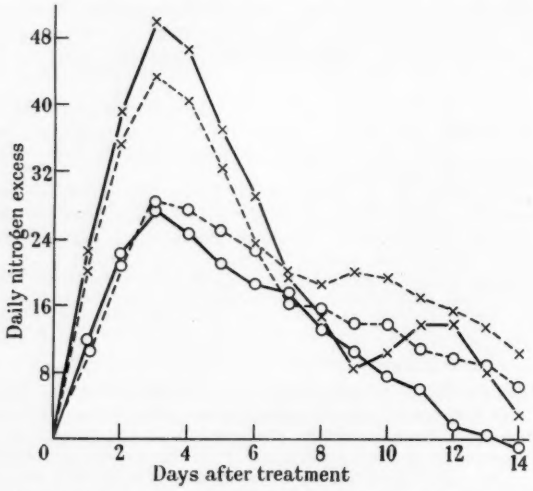

FIGURE.—Daily excess of urinary nitrogen over basal level in mg. nitrogen/100 g. body weight.
×——× fracture + cortisone 25 mg.
× - - - × cortisone 50 mg.
⊙——⊙ fracture.
⊙ - - - ⊙ cortisone 25 mg.

TABLE I.—*Excess Urinary Nitrogen Excretion in Response to Different Treatments.*

			Treatment.	Increase in urinary N*	Approximate S.E of difference between means.†
Group I	Exp. 1	A	Cortisone 25 mg.	245	± 58
		B	Fracture	210	
		C	Cortisone 50 mg.	321	
		D	Fracture + cortisone 25 mg.	290	
	„ 2	A	1 Incision	190	± 41
		B	Dummy tablet	179	
		C	Cortisone 25 mg.	258	
	„ 3	A	1 Incision	86	± 40
		B	2 Incisions	140	
		C	Cortisone 50 mg.	268	
		D	„ 75 mg.	288	
	„ 4	A	3 Incisions	174	± 66
		B	Cortisone 75 mg.	338	
		C	„ 75 mg.	383	
		D	Fracture + cortisone 50 mg.	370	
Group II	„ 5	A	Sham-operated + fracture	253	± 74
		B	Adrenalectomized + fracture	1	
			Each group maintained on 0·9 per cent saline		
	„ 6	A	Sham-operated + fracture	171	± 51
		B‡	Sham-operated + fracture + oral cortisone	168	
		C‡	Adrenalectomized + fracture + oral cortisone	124	

* The increase in the urinary nitrogen excretion in 14 days over the basal level expressed as mg./100 g. initial body wt.

† All standard errors here and in Table II are based on approximately 10 degrees of freedom.

‡ Maintained throughout on oral cortisone acetate, 0·8 mg./100 g. initial body wt. daily.

Urinary Nitrogen.

Group 1

Exp. 1.—The effects of implanting a 25 mg. tablet of cortisone acetate and of fracture of a femur were similar in magnitude as measured by the resulting increase in urinary nitrogen excretion, the mean excretion/100 g. body wt. being 245 mg. and 210 mg. over 14 days with an average of 228 mg. When either a fracture or the implantation of a second 25 mg. tablet was effected simultaneously with the implantation of one 25 mg. tablet, the resulting responses were again similar (290 mg. and 321 mg. respectively, with an average of 306 mg.). The response to the additional 25 mg. cortisone was 78 ± 41 mg. additional urinary nitrogen excretion.

Exp. 2 was performed to find out to what extent the increase in the urinary nitrogen excretion found at the lowest level of treatment was due to the effect of incision or to the presence of the tablet.

The animals subjected to incision and to the dummy tablet showed a resultant increase in urinary nitrogen excretion (average 184 mg.) and the 25 mg. cortisone acetate implantation gave a value of 258 mg. The difference was 74 mg. with S.E. ± 35 mg. This did not quite attain significance at the 5 per cent level. It thus appeared that under the conditions of this experiment most of the increase in urinary nitrogen excretion following implantation of cortisone was due to incision ;

TABLE II.—*Size of Groups, Food and Nitrogen Intakes and Regression of Response on Basal Urinary Nitrogen Excretion.*

			Size of group.	Body weight (g.). Average of 3 days before operation.	Body weight (g.). 14 days after operation.	Daily food and nitrogen intakes g./100 g. initial body wt. (nitrogen in brackets). 3 days pre-operative period.	Daily food and nitrogen intakes g./100 g. initial body wt. (nitrogen in brackets). 14 days post-operative period.	Mean.	b* and its S.E.
Group I	Exp. 1	A	6	306	331	7·08 (0·203)	7·12 (0·204)	7·4	−4·5 ±2·23
		B	6	311	344	7·64 (0·219)	7·64 (0·219)		
		C	6	307	334	7·62 (0·218)	7·42 (0·213)		
		D	5	298	320	7·20 (0·206)	7·20 (0·206)		
	„ 2	A	6	274	288	7·16 (0·212)	7·16 (0·212)	7·1	− 5·4 ± 1·66
		B	6	294	308	7·05 (0·209)	7·05 (0·209)		
		C	6	290	302	7·22 (0·214)	7·22 (0·214)		
	„ 3	A	4	331	354	6·38 (0·202)	6·38 (0·202)	6·3	−10·4 ±1·75
		B	4	337	353	6·39 (0·202)	6·39 (0·202)		
		C	4	352	366	6·42 (0·203)	6·42 (0·203)		
		D	4	334	338	6·18 (0·196)	6·18 (0·196)		
	„ 4	A	5	322	339	7·12 (0·207)	7·12 (0·207)	6·9	−9·0 ±3·76
		B	5	308	307	6·86 (0·199)	6·86 (0·199)		
		C	5	311	300	6·88 (0·200)	6·85 (0·199)		
		D	5	314	306	6·72 (0·195)	6·72 (0·195)		
Group II	„ 5	A	7	344	352	6·12 (0·189)	6·09 (0·188)	5·9	−11·4 ±3·22
		B	7	339	318	5·74 (0·177)	5·65 (0·174)		
	„ 6	A	6	345	349	6·16 (0·177)	6·16 (0·177)	6·1	−7·9 ±1·65
		B	6	376	384	6·18 (0·178)	6·18 (0·178)		
		C	6	348	335	5·89 (0·169)	5·84 (0·168)		

* b is the regression coefficient of the response to treatment upon the level of basal urinary nitrogen excretion measured in mg. response nitrogen/mg. basal urinary nitrogen.

TABLE III.—*Body Weights before and after Adrenalectomy or Sham Operation.*

		Treatment.	Body weight (g.). Initial.	Body weight (g.). 14 days after operation.
Exp. 5	A . Sham-operated B . Adrenalectomized	maintained on 0·9 per cent saline from day of operation	332 338	345 338
Exp. 6	A . Sham-operated B . Sham-operated C . Adrenalectomized	maintained on oral cortisone from day of operation . .	335 360 339	348 377 348

the additional excretion due to the cortisone itself was 74 mg. The effect of a second 25 mg. was similar to that of the first. Further, the response to fracture is, from past experience, in part due to the incision during induction of fracture.

Exp. 3 showed that two incisions caused 56 mg. more nitrogen excretion than one, and that the implantation of three tablets caused a response 20 mg. greater than that from two. Neither of these differences approached significance.

Exp. 4 was performed to decide whether the effect of fracture + 50 mg. cortisone was comparable with 75 mg. cortisone alone ; *i.e.*, whether at this high level of stimulus 25 mg. cortisone was still equivalent to fracture ; a further treatment group was also introduced to observe the effect of three incisions.

Groups B, C, and D (Table I) had all considerably greater responses than Group A, but were very similar to one another, suggesting that the effect of fracture is similar to that of 25 mg. cortisone at this highest superimposition level. Whether two or three incisions were used for implantation made little difference.

Group II.

These experiments were designed to investigate the response of adrenalectomized rats maintained on oral cortisone.

Exp. 5.—The effect of fracture on nitrogen excretion in the sham-operated group was similar to that already obtained, whereas adrenalectomy appeared to prevent the characteristic response to injury.

Exp. 6.—This was designed to investigate the " permissive " or " conditioning " effect of cortisone acetate on response to fracture in adrenalectomized rats. There were no significant differences between the treatments, suggesting that the feeding of cortisone compensated for removal of the adrenals in so far as these effects were concerned. The cortisone-fed sham-operated rats had as great a response to fracture as the sham-operated rats which received no cortisone.

In all six experiments covariance analyses were carried out on the basal level of nitrogen excretion and on the food intake. It was generally found that the regression on the basal nitrogen level was the better of the two and that no additional information was obtained by including the regression on food intake. Differences in the basal nitrogen level were taken into account in calculating the responses as given in Table I.

In addition to these major effects we also found that in each experiment the response depended to some extent on the basal urinary nitrogen level (Table II) and that this modifying effect on response varied between experiments in a way which seemed to depend on food intake. Animals with a low basal level of urinary

nitrogen responded more to trauma than those with a high basal level ; *i.e.*, there was negative regression between response and basal. This regression differed from one experiment to another. Where the food intake level was high, the effect of the basal level of urinary nitrogen excretion on the response was smaller than in the experiments with low food intake level. This relationship is being further investigated.

Growth Rate.

Group I.

Exp. 1–4.—Both implantation of 25 mg. cortisone acetate and fracture caused slight recessions in growth, but these were offset in the later stages (Table II). Neither one, two nor three incisions, nor the presence of the dummy tablet altered growth rate. The rats having treatments fracture + cortisone 25 mg. or cortisone 50 mg. just maintained their body weight over the first 6–8 days and then gained weight steadily until the end of the experiment. In the groups fracture + cortisone 50 mg. and cortisone 75 mg. the animals lost weight throughout the 14-day period. There was no reduction in food intake to account for this loss as the food intake and its nitrogen content did not vary between the pre- and post-operative periods in any group within any of the experiments in Group I.

Group II.

Exp. 5.—The animals in the sham-operated group increased in body weight during the 14 days following operation. The adrenalectomized saline-maintained groups just maintained their initial body weight over the same period (Table III). Following fracture the sham-operated rats continued to gain in weight, while those in the adrenalectomized group steadily lost weight (Table II), although food intake was not markedly reduced.

Exp. 6.—In groups A, B, and C (Table III) all animals gained weight after adrenalectomy or sham operation. Following fracture both sham-operated groups showed a very slight gain in weight. The adrenalectomized group lost weight. There was no change in food intake following fracture adequate to account for this loss.

DISCUSSION.

These experiments show that nitrogen loss in the urine after fracture of a long bone and after subcutaneous implantation of cortisone follow similar courses. In both cases the nitrogen is lost mainly as urea.

At first sight the evidence would seem to indicate that the effect of a trauma such as the fracture of a long bone on nitrogen excretion is probably due to the liberation of active adrenal cortical hormone equivalent in effect to that resulting from the implantation of 25 mg. cortisone acetate, but the observations of Ingle, Ward and Kuizenga (1947), confirmed by Ingle, Meeks and Thomas (1951) and now by ourselves, show that the adrenal cortex is itself unnecessary, provided there is sufficient circulating hormone present.

Noble and Toby (1946–48) found that the urinary nitrogen response to trauma was unaffected when only one adrenal was removed. Bilaterally adrenalectomized animals showed on the average no elevation of urinary nitrogen after trauma, although a response occurred in a few animals, but the blood N-PN was consistently increased in those not showing increased nitrogen output. In their

adrenalectomized animals treated with " maintenance " doses of cortical extract no excess urinary nitrogen was found after trauma comparable to that found in intact rats. The amount of cortical extract used did not affect the nitrogen excretion of untraumatized adrenalectomized animals.

Our results might be explained as Ingle (1951) and Selye (1954) have suggested, on the basis of " essentiality " or " permissibility," which is an extension of the hypothesis of Selye and Collip (1936) in regard to their observations on the anterior pituitary. This " essentiality " assumes that the stimulus acts on the target organ or tissue in the presence, but not in the absence, of the endocrine gland or its hormone. It further assumes that the stimulus acts directly, but only if the target organ or tissue is maintained in a responsive condition by constant hormonal impulses received from the gland. Selye and Horava (1953) prefer the word " conditioning," since this obviates the idea that the factor is inert, and reinforces the view that it is *not* an all-or-none effect or a sensitization effect. They point out that in all instances of hormone-overcharge the manifestations of the hyperhormonal condition are quantitatively dependent just as much on the blood-level of the apparently responsible hormone as on the conditioning factors which determine sensitivity to it. By way of comparison Selye (1954) points out that traumatic shock produces hyperglycaemia in the intact, but hypoglycaemia in the adrenalectomized rat, and that if an adrenalectomized rat is maintained on threshold doses of adreno-cortical extracts (which in themselves cause no hyperglycaemia) it can again respond to surgical shock normally, that is by a marked rise in blood sugar. It is therefore concluded that in such an animal the metabolic changes consequent on trauma produce favourable conditions for the manifestation of the hyperglycaemic effect of glucocorticoids.

The recent observations of Speirs and Meyers (1949), Speirs (1953), and Speirs and Sullivan (1953) are of particular interest for they indicate that a large proportion of adrenalectomized mice possess adrenal accessories which are capable of responding to an injection of adrenaline by releasing sufficient C_{11}-oxysteroids to induce a definite eosinopenia. The work of Swingle, Maxwell, Ben, Fedor, Baker, Eisler and Barlow (1954) supports the view that cortical accessories take over to a limited extent the function of the glands, for example for maintenance on saline drinking water. Swingle *et al.* hold that although the prevalence of accessory cortices and their functional development in the adrenalectomized rat maintained by various means must be conceded, it seems probable that only the results of the salt maintenance experiments can be satisfactorily accounted for on the basis of accessory adrenal cortical tissue, a finding in agreement with the ability of our adrenalectomized animals in Exp. 5 to maintain themselves on saline. Although Speirs and Sullivan (1953) noted an eosinopenia in adrenalectomized mice following injection of adrenaline we observed no rise in urinary nitrogen excretion following fracture in our adrenalectomized rats unless maintained on a level of cortisone which was itself insufficient to produce a response. It is conceivable, however, that this maintenance level permits the accessory tissue to respond to trauma and so to take over the rôle of the intact adrenal, thus allowing the full response to injury in terms of nitrogen excretion.

Is this loss of nitrogen following trauma or cortisone due to a catabolism of body protein or to an antianabolic effect on absorbed food amino acids leading to the earlier elimination of their constituent nitrogen ? One of us (Cuthbertson, 1932, 1942) considered that following an initial period of depressed metabolism the extent

of the overall loss following trauma was more general than local and was in man sometimes so great as to preclude an antianabolic effect as the primary cause.

In an investigation on the effect of changing the level of protein in the diet at the time of fracture, Munro and Chalmers (1945) found that the influence of an adequate protein intake on fracture metabolism was lost as soon as the protein intake was reduced.

Albright (1942) first suggested that the C_{11}-oxysteroids were perhaps concerned with an antianabolic action rather than a catabolic one on protein. This hypothesis that cortisone has an antianabolic effect is accepted by Clark (1953) who found that the uptake of ^{15}N when fed as glycine for one or two days was less in cortisone-treated animals than in controls.

On the other hand, the experiments of White and Roberts (1950) and Roberts (1951, 1952) support the view that the mobilization of tissue proteins is an important function of the adrenal cortical hormones and suggest that the effects of these hormones on protein metabolism are produced primarily by labilization of the tissue proteins. In his most recent paper Roberts (1953) has shown that the end result of the labilization and the translocation of protein in the body due to pituitary-adrenal cortical activation may be local or systemic protein anabolism or catabolism, depending on the tissue requirements of the moment. The labilization of tissue protein was found to be directly under the control of the adrenal cortex. Evidence is not yet available to determine whether the translocation of tissue protein was directly or secondarily influenced by the hormonal secretion of the adrenal cortex.

Roberts (1953) further suggests that this labilization of tissue proteins may constitute the " permissive " action of the adrenal cortical steroids on protein metabolism described by Ingle *et al.* (1947). Ingle (1951) has suggested that hormones of the adrenal cortex serve mainly to permit homeostatic adjustments of the body to disturbing environmental influences. It seems probable from the data presented that these hormones increase the availability of tissue protein and other materials for the tissues.

It has already been suggested that anabolic and catabolic processes may possibly occur at the same time in different parts of the body. Roberts suggests that the latter situation appears to exist in the traumatized animal, in which undamaged tissues supply protein for the repair of damaged structures and that these proteins appear to be translocated to the damaged site as specific serum proteins. Also using labelled nitrogen, Roberts (1953) found that the release of protein to a serum medium by rat liver and spleen *in vitro* was enhanced by previous treatment with adrenal extract or ACTH. The experiments of Fischel, Stoerk and Bjørneboe (1951) on the rate of disappearance of passively administered antibody from the circulation of cortisone-treated animals might be held to support the antianabolic theory. But such evidence requires careful interpretation as passively administered antibody protein may not be subjected to the usual catabolic mechanisms.

Although from a qualitative approach the extra N excreted by the fractured rat might sometimes be due to an antianabolic effect inducing an acceleration in the elimination of nitrogen, nevertheless previous observations on man and the rat indicate that the scale of loss is greater than can be accounted for by such an accelerated elimination alone.

The evidence as a whole supports the belief that there is a general antianabolic

effect, particularly during the early post-injury period, but that thereafter the rise in the urinary excretion of nitrogen is due to a catabolic response which involves a loss of body substance in the well nourished animal, as earlier described by Munro and Cuthbertson (1943).

SUMMARY.

Experiments were made to compare the effect on urinary nitrogen excretion in the rat of subcutaneous implantation of 25 mg. cortisone acetate with fracture of a femur by open operation. The " permissive " effect of a maintenance level of fed cortisone acetate was also investigated in the adrenalectomized animal.

The effect of implantation of 25 mg. cortisone acetate was similar in magnitude and shape of response curve to that of fracture whether these treatments were applied alone, or along with implantation of 25 or 50 mg. cortisone.

Most of the response to fracture or the implantation of 25 mg. cortisone in this series of experiments appeared to result from the necessary incision.

The effect of a second tablet was similar in magnitude to that of the first, but the additional response due to a third tablet was very small.

Adrenalectomized animals, able to maintain themselves on saline, showed no response to fracture, but when such animals were fed cortisone daily at a maintenance level the characteristic response to fracture was again obtained.

The magnitude of response measured also appeared to depend on the basal urinary nitrogen excretion. The extent of the dependence varied between experiments and appeared to be affected by the general level of food intake.

The animals less severely treated continued to gain in weight. Others maintained their weight, or lost weight, according to the severity of treatment.

The authors wish to express their indebtedness to the Medical Research Council for their gift of cortisone acetate (" Cortone," Merck).

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THE HISTOCHEMISTRY OF THE PROSTATE IN NORMAL AND IN CASTRATED AND HORMONE-TREATED MICE AND OF PROSTATIC HOMOGRAFTS EXPOSED TO 20-METHYL-CHOLANTHRENE.

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Received for publication August 11, 1954.

THE mouse prostate consists of paired anterior (coagulating glands), ventral and dorsal lobes, and a small single median gland (Deanesly and Parkes, 1933). In this study we have investigated the distribution in the various lobes of the histochemical reactions for alkaline and acid phosphatases, lipase, α-esterases, succinic dehydrogenase, xanthine oxidase, periodic acid-Schiff reacting substances, SS and SH groups, and lipids.

Changes in these histochemical reactions were studied in the mouse prostate following castration and treatment with various hormones, and also in normal prostate homografts and homografts treated with 20-methylcholanthrene.

MATERIALS AND METHODS

Male mice of an inbred strain (Strong A), 2–5 months old, were used. They were killed by a blow on the neck. The tissues were immediately removed and placed in the following fixatives: 10 per cent formalin-saline, chilled acetone, formalin-cobalt nitrate, 1 per cent trichloracetic acid in 80 per cent alcohol. In addition, very small pieces of the fresh tissue were incubated in suitable substrates for the demonstration of succinic dehydrogenase and xanthine oxidase.

Acetone-fixed tissues were cleared in methyl benzoate and embedded in paraffin. Sections were cut at 7μ and subjected to techniques for alkaline and acid phosphatase (Gomori, 1939; 1950); lipase (Gomori, 1945); α-esterase (Gomori, 1952) and the periodic acid-Schiff reaction (Hotchkiss, 1948; McManus, 1946). The formalin-cobalt nitrate fixed tissues were used for the demonstration of the Golgi apparatus by Da Fano's (1920) method. The trichloracetic acid-alcohol fixed material was dehydrated through alcohols, cleared in methyl benzoate and embedded in paraffin. Sections were cut at 10μ, and the method of Barrnett and Seligman (1952) for the demonstration of protein-bound sulphydryl groups was employed. Frozen sections cut from the formalin-saline fixed tissues were stained with Sudan Black. Succinic dehydrogenase was demonstrated in the fresh tissue by the method of Seligman and Rutenberg (1951; see also Bourne and Malaty, 1953). Xanthine oxidase was demonstrated by the method described by Bourne (1953).

RESULTS.

Histochemistry of Normal Mouse Prostate.

Alkaline phosphatase.

In all three lobes there was a positive reaction in the basement membrane of the acini, the endothelium of small vessels and the sheaths of smooth muscle fibres in the stroma. This agrees with other authors. Bern (1949), however, using

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longer incubation periods, found a positive reaction in the secretion and epithelium of the ventral lobe. Stromal distribution of phosphatase in male accessory glands has also been recorded by Dempsey, Greep and Deane (1949) for the seminal vesicles of the rat, and by Atkinson (1948) for the mouse.

Acid phosphatase.

Positive reactions, mainly in epithelium, were found in all lobes. Nuclei were moderately positive, the strongest reaction being given by the region of the Golgi apparatus. The localization of phosphatases in the Golgi region was first recorded in gut epithelial cells by Bourne (1943), with the alkaline phosphatase reaction. Similar results were obtained by Emmel (1945) and Deane and Dempsey (1945), who also found acid phosphatase in the Golgi region of epithelial cells : in the gut, seminal vesicles and uterine mucosa.

Periodic acid-Schiff reaction (P.A.S.).

Basement membranes, endothelium of capillaries and luminal secretion were positive. Epithelial cells also showed positive intracytoplasmic granules, some aggregated near the free border, others in the Golgi region. (See also Leblond, 1950 ; Arcadi, 1952.)

Lipase and α-esterase.

The lipase reaction was negative and the α-esterase positive only in the median lobe.

Succinic dehydrogenase.

A positive granular cytoplasmic reaction was found in the epithelial cells.

Xanthine oxidase.

Positive granular and diffuse cytoplasmic reactions were obtained. Some granules resembled mitochondria in form.

SH and SS groups.

In the ventral lobe, moderate reaction was seen in the basement membrane and epithelial cells. This was stronger in these sites in the anterior lobe where the secretion reacted intensely.

Sudan Black.

Positive reaction was seen in the epithelial cells of the three lobes, localized in the Golgi region and closely resembling the Golgi apparatus as seen with silver technique.

Histochemical Changes in the Mouse Prostate after Castration and following Administration of Androgenic and Oestrogenic Substances.

Moore, Price and Gallagher (1930) showed that castration and sex hormone administration cause cytological changes in the prostate of the rat, and Horning (1947) showed that these procedures also affect the Golgi apparatus in the mouse prostate.

Histochemical changes in the mouse prostate were therefore investigated in the same conditions. Strong A mice about 4 months old were used.

The first group (18 animals) was killed at 4–22 days after castration. A second group (12 animals) received an implantation of 10 mg. testosterone propionate 13 or 22 days after castration, and were killed 8–21 days after the implant. Another group (24 animals) had 10 mg. diethyl stilboestrol implanted; 15 were killed 4–20 days after the implant. In the remaining animals of this group the pellet of stilboestrol was removed 18 days after it was originally implanted and observations were made at 12, 17 and 21 days thereafter. Control prostates were carried through the same histochemical procedures with the experimental prostates.

Effects of castration.

Hyperplasia of the fibromuscular stroma (moderate after 8 days, increasing with duration of castration) was found and the epithelial cells lining the acini proliferated into several layers without however showing squamous metaplasia. These changes were more marked in the anterior lobes.

The histochemical reactions at different intervals after castration are shown in Table I.

Implantation of testosterone propionate in the castrated animal.

Implantations were made 13 or 22 days after castration and the observations recorded in Table II were made 8 to 21 days later.

The effect of stilboestrol on the normal prostate.

Each animal was implanted subcutaneously with 10 mg. diethyl-stilboestrol. Observations were made 4, 8, 12, 14 and 20 days after implantation.

From the 4th day the fibromuscular stroma increased, and seemed to compress some of the acini in which varying degrees of epithelial hyperplasia were present. Thus the walls of the acini, normally lined by one layer of cuboidal or cylindrical cells, showed several layers of epithelial cells which tended to become polyhedral. Such changes were more prominent in the anterior lobe.

The histochemical changes in these animals are set out in Table III.

Changes after the removal of the stilboestrol implant.

This group also received 10 mg. diethyl-stilboestrol but the implants were removed after 18 days. Observations were made 12, 17 and 21 days later, as recorded in Table IV.

Histochemical Changes in Normal and Methylcholanthrene-treated Homografts.

For this section of the work Strong A male mice 4 or 5 months old were used. Under nembutal anaesthesia the anterior and ventral lobes of the prostate were removed. In one series small pieces of anterior and ventral lobes were implanted subcutaneously by means of a Bashford needle in the right and left axillary regions respectively; in a second series the prostatic tissue was impregnated with a few crystals of 20-methylcholanthrene before its implantation in the subcutaneous tissue of the host, using the method described by Horning (1946). The animals bearing the grafts were killed at different periods ranging from 1–60 days. The

TABLE I.—*Histochemical Reactions of the Mouse Prostate after Castration.*

Days after castration.	P.A.S. reaction.	Acid phosphatase.	Alkaline phosphatase.	Golgi apparatus.
4	No change	Positive reaction in Golgi region became less compact and filaments resembling mitochondria became visible in this region.	Positive reaction in basement membrane, normally forming a continuous layer around the acini, became slightly discontinuous and less intense.	No change.
8	Decreased reaction in cytoplasmic granules and in the luminal secretion	Above changes accentuated.	Above changes accentuated.	No change.
12–14	Further decrease of reaction in above sites.	Positive reaction in Golgi region consisted of filamentous bodies similar to mitochondria. Positive reaction in basal cytoplasm.	Above changes accentuated.	The apparatus consisted of a less dense network, slightly fragmented.
21–22	Reaction almost completely negative in epithelium and lumen of acini and decreased in basement membrane. Sheaths of normal and hyperplastic smooth muscle positive.	Reaction in Golgi region limited to very scarce mitochondria-like bodies. Moderate nuclear reaction throughout the experiment.	Reaction in basement membrane still positive, but marked discontinuity and loss of intensity. Sheaths of smooth muscle strongly positive.	The normally compact network became loose and fragmented.

TABLE II.—*Histochemical Changes in the Mouse Prostate after Castration followed by Testosterone Implantation.*

P.A.S. reaction.	Acid phosphatase.	Alkaline phosphatase.	Golgi apparatus.
There was a gradual return to normal of the positive reaction in cytoplasmic granules, in the secretion and basement membrane. The sheaths of smooth muscle were also positive.	The reaction in the epithelium regained its normal aspect, showing a strong reaction in the Golgi region, in the form of a compact body. The positive reaction in the nuclei remained unchanged, as well as that which developed at the basal part of some cells.	Rapid return to normal as indicated by the positive reaction in the basement membrane becoming again an uninterrupted layer surrounding the acini.	Normal aspect quickly attained, the apparatus appearing as a compact network in the supranuclear region.

grafted tissue was fixed in chilled acetone on removal and treated as described earlier for various histochemical techniques.

Changes in untreated grafts.

The anterior and ventral lobes behaved differently after transplantation and will therefore be treated separately.

Anterior lobe.—First week : there was a disappearance of the positive P.A.S. secretion from the lumina. Only a few epithelial cells in the periphery of the graft showed P.A.S.-positive cytoplasmic granules. Except in a few peripherally situated acini the acid phosphatase reaction became more feeble and finally disappeared from the nuclei and Golgi region, and the alkaline phosphatase reaction in the basal membrane became blurred and discontinuous. In many acini (especially in the centre of the graft) the epithelium was detached and lay within the lumen where, together with some secretion and inflammatory cells, it formed a necrotic mass giving positive P.A.S. and acid phosphatase reactions. The stroma of the graft showed an inflammatory reaction, with oedema and infiltration by polymorphonuclear and mononuclear cells. The epithelial cells in the meantime had become flattened but by the 5th–6th days they started becoming taller, and even showed some degree of proliferation, giving rise to buds which might later differentiate into new acini. Second week : the same conditions existed at the centre of the graft but at the periphery the epithelial cells of the graft showed an increase of P.A.S.-positive granules in the cytoplasm and a stronger reaction for acid phosphatase in the nuclei and cytoplasm. In the latter the positive reaction was localised in the Golgi region. The alkaline phosphatase reaction was still negative in the centre of the graft and weakly positive in the basement membrane of some of the peripheral acini. The stroma had the same inflammatory reaction. End of the third week : the changes in the grafts produced prostatic tissue which very much resembled the normal. The inflammatory reaction had nearly disappeared from the stroma; there was a general increase in connective tissue represented by the larger amounts of ground substance, reticulin fibrils and proliferating fibroblasts, some of which contained P.A.S.-positive granules. The difference between the peripheral and central parts of the grafts was less striking, the latter showing a greater amount of connective tissue. The epithelial cells of both portions of the grafts showed abundant P.A.S.-positive granules, some

TABLE III.—*Effects of Stilboestrol on the Histochemistry of the Mouse Prostate.*

Days after implantation of stilboestrol.	P.A.S reaction.	Acid phosphatase.	Alkaline phosphatase.	Golgi apparatus.
4	No change in cytoplasmic granules or secretion. Stronger reaction in basement membrane.	Positive reaction in nuclei and Golgi region. Latter less compact than normal.	No change.	Normal.
8	Moderate decrease in cytoplasmic granules and luminal secretion. Strong reaction in basement membrane and adjacent reticulin fibres.	Positive reaction in nuclei. In the Golgi region the reaction was less compact, showing filamentous structures resembling mitochondria.	The reaction in the basement membrane discontinuous and less intense.	Still appeared normal.
12–14	Accentuation of preceding changes.	Nuclei remained positive. In the Golgi region the reaction consisted of filamentous and granular structures.	Accentuation of above changes.	A less dense network, which appeared to be reduced in size.
20	The reaction in the cytoplasmic granules and in the luminal secretion greatly reduced. Strong reaction in basement membrane, nearby reticulin fibres and sheaths of smooth muscle.	Nuclei still positive. Reaction in Golgi region limited to few granules and filaments.	The discontinuity and loss of intensity of the reaction in the basement membrane more marked, although the sheaths of smooth muscle gave a strong positive reaction.	A loose structure formed by fragmented filaments and granular material, with some reduction in size.

TABLE IV.—*Histochemistry of the Mouse Prostate after Withdrawal of Stilboestrol.*

Days after removal of stilboestrol.	P.A.S. reaction.	Acid phosphatase.	Alkaline phosphatase.	Golgi apparatus.
12	Positive reaction in few cytoplasmic granules; very scarce or absent in luminal secretion. Normal reaction in basement membrane and adjacent reticulin fibres.	Positive reaction in nuclei. In the Golgi region it still consisted of granular and filamentous structures. Also positive reaction in the base of cells.	Reaction in the basement membrane still discontinuous and less intense.	Still reduced in size and mainly composed of loose network.
17	Normal positive reaction in cytoplasmic granules; still scarce in luminal secretion especially in the anterior lobe.	Positive reaction in nuclei. In the Golgi region the reaction showed a compact body resembling the Golgi apparatus.	In the basement membrane the reaction became continuous and regained its normal intensity.	More compact and larger in size.
21	Normal positive reaction in cytoplasmic granules; increased in the luminal secretion but still scarce in the anterior lobe.	The reaction appeared to be normal, both in nuclei and Golgi region.	Normal reaction in the basement membrane and endothelium of small blood vessels. Also positive in sheaths of smooth muscle.	Normal appearance.

accumulated in the Golgi region. The acid phosphatase reaction reappeared in the nuclei and the Golgi region, and the alkaline phosphatase reaction in the basement membrane as a sharp uninterrupted line surrounding the acini. At this stage the lumina of the acini were filled with P.A.S.-positive secretion. Fourth week onwards: the grafted tissue appeared very similar to the normal prostate *in situ*, both histologically and histochemically. After some time, however, the acini tended to become distended with secretion and the epithelial cells lining them became flattened.

Ventral lobe.—Similar changes occurred in the ventral lobe, but it differed from the anterior lobe in that the normal histochemical picture did not return even after 7 weeks, and there was little sign of any return of secretion in the lumina.

Changes in grafts treated with 20-methylcholanthrene.

All of these grafts, which became vascularized and survived, underwent squamous cell metaplasia. There was a proliferation of the acinar epithelium which showed a stratification of several layers instead of the usual single layer of columnar cells. These proliferating cells, which finally obliterated the lumen of the acini, underwent keratinization and formed large masses of keratin. While this process, which was sometimes completed before the 10th day, was taking place, changes in the histochemical pattern of the acini occurred. This was marked by the gradual disappearance of the intracellular and intra-acinar P.A.S.-positive material, while the basement membrane became thicker and remained strongly positive. The acid phosphatase reaction became very feeble in both nuclei and cytoplasm (particularly in the Golgi region). On the other hand, the keratohyalin granules showed a strongly positive acid phosphatase reaction. The alkaline phosphatase, normally localized in the basement membranes, disappeared very early in the process of squamous metaplasia.

Some grafts underwent malignant transformation with the production of squamous cell carcinomas, and in these the acid and alkaline phosphatase reactions disappeared, but the basement membranes surrounding the epithelial cords gave a positive P.A.S. reaction.

DISCUSSION.

Our results have shown that the distribution of the acid and alkaline phosphatase and the P.A.S. reactions are similar in the three lobes, which are also alike in giving no lipase reaction. However, the anterior lobe shows a greater reaction for SS and SH groups than the ventral lobe. Bern (1949) and Stafford, Rubinstein and Meyer (1949) have described alkaline phosphatase in the epithelium of the ventral lobe, whereas in our preparations the reaction was restricted to the stroma and basement membranes. Difference in the length of incubation may be responsible for this, as the above authors used longer incubation times. The stromal distribution of alkaline phosphatase has been found in other male accessory sex organs, *e.g.*, in the seminal vesicle of the rat (Dempsey *et al.*, 1949) and mouse (Atkinson, 1948). In the human prostate alkaline phosphatase is usually localized in the stroma, although Gomori (1941) and Kabat and Furth (1941) have described some reaction in the epithelial cells and in the secretion.

Acid phosphatase, however, is an epithelial enzyme in the prostate, and has been recorded in the prostatic epithelium and secretion of the dog by Gomori (1941)

EXPLANATION OF PLATES.

FIG. 1.—Golgi preparation of anterior lobe of prostate in normal mouse, showing localization of the apparatus in distal region of cell. × 1000.

FIG. 2.—Golgi preparation of anterior lobe of prostate of castrated mouse showing flattening of cells, reduction in size and less compact form of Golgi apparatus. × 1000.

FIG. 3.—Golgi preparation of anterior lobe of prostate of castrated mouse implanted with testosterone, showing normal form and position of Golgi apparatus. × 300.

FIG. 4.—Acid phosphatase preparation of anterior lobe of prostate of normal mouse showing localization of main part of reaction in the Golgi region. Very little reaction is given by the nuclei. × 1000.

FIG. 5.—Acid phosphatase preparation of anterior lobe of prostate of castrated mouse. There is no reaction in the Golgi region, but a strong reaction is given by the nuclei. × 1000.

FIG. 6.—Acid phosphatase preparation of anterior lobe of prostate of castrated mouse implanted with testosterone. Return of reaction to Golgi region but reaction still present in nuclei. × 1000.

FIG. 7.—Acid phosphatase preparation of anterior lobe of prostate of mouse implanted with stilboestrol. Absence of reaction in Golgi region but nuclei positive. × 1000.

FIG. 8.—Acid phosphatase in a 7-day graft of anterior lobe of prostate of mouse. Great reduction of reaction. × 300.

FIG. 9.—Acid phosphatase preparation of graft of anterior lobe of mouse prostate treated with methylcholanthrene before implantation. Squamous metaplasia. Very faint reaction in nuclei, otherwise completely negative. × 300.

FIG. 10.—Acid phosphatase preparation of graft of anterior lobe of mouse prostate treated with methylcholanthrene. Squamous cell carcinoma. Faint reaction in nuclei, otherwise completely negative. × 300.

FIG. 11.—P.A.S. reaction for polysaccharides. Anterior lobe of normal mouse prostate. Positive reaction by acinar cells. × 1000.

FIG. 12.—P.A.S. reaction for polysaccharides. Anterior lobe of prostate of castrated mouse. Great reduction of reaction in cells. × 300.

FIG. 13.—P.A.S. reaction for polysaccharides. Anterior lobe of prostate of castrated mouse implanted with testosterone. Lumen filled with positive reacting secretion (black in figure) and cells with positive reaction. × 700.

FIG. 14.—P.A.S. reaction for polysaccharides. Graft of anterior lobe of mouse prostate. Great reduction of intensity of reaction in cells and absence of secretion. × 300.

FIG. 15.—P.A.S. reaction for polysaccharides. Anterior lobe of prostate of mouse with implant of stilboestrol. Great reduction of reaction in cells. Some reaction persists in stroma. × 300.

FIG. 16.—P.A.S. reaction for polysaccharides. Graft of anterior lobe of mouse prostate treated with methylcholanthrene. Squamous metaplasia. Complete absence of reaction in cells. Some positive reaction in stroma. Apparent positive reaction is due to haematoxylin staining. × 500.

FIG. 17.—P.A.S. reaction for polysaccharides. Graft of anterior lobe of mouse prostate treated with methylcholanthrene. Squamous cell carcinoma. Cells completely negative. Small reaction persists in stroma in parts. × 300.

FIG. 18.—Alkaline phosphatase reaction in anterior lobe of normal mouse prostate. Reaction completely restricted to stroma and basement membranes. × 300.

FIG. 19.—Alkaline phosphatase reaction in anterior lobe of prostate of castrated mouse. Great reduction of intensity of reaction. × 300.

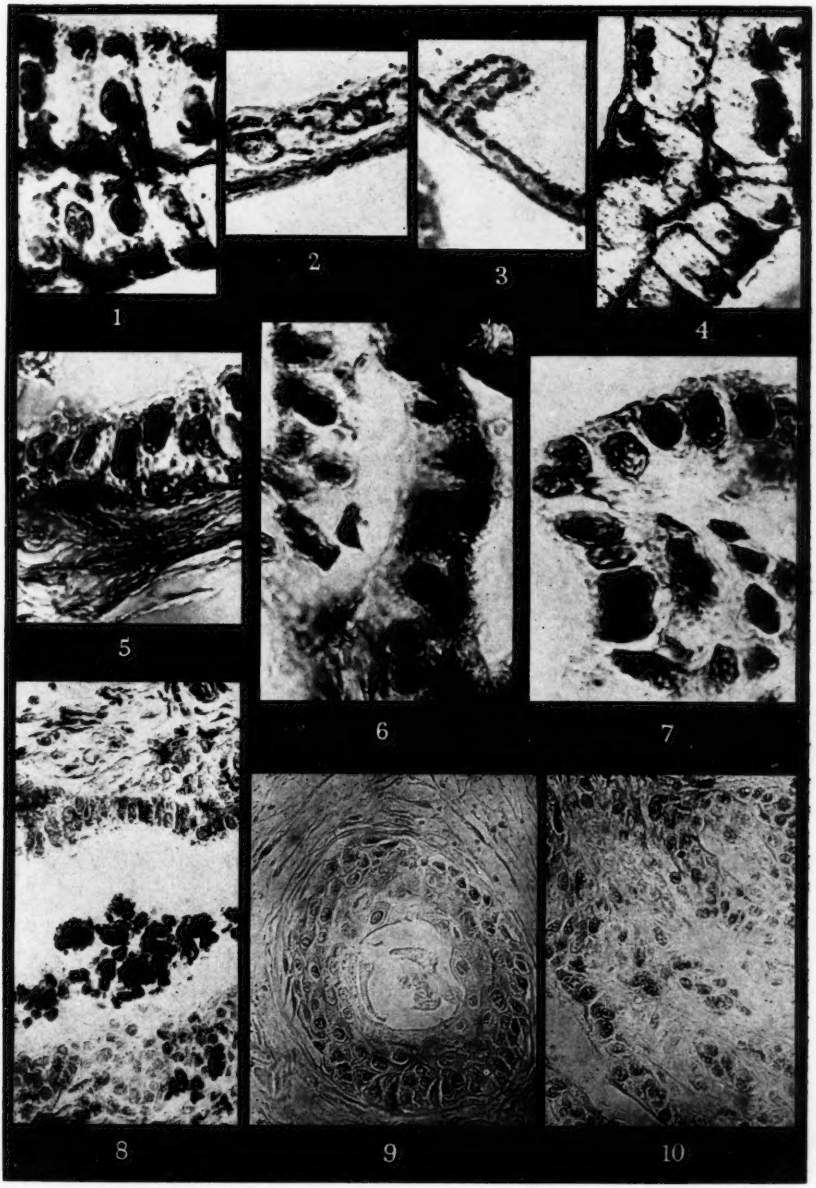
FIG. 20.—Alkaline phosphatase reaction in anterior lobe of prostate of castrated mouse implanted with testosterone. Normal reaction. × 300.

FIG. 21.—Alkaline phosphatase reaction in anterior lobe of prostate of mouse implanted with stilboestrol. Decreased intensity of reaction. × 300.

FIG. 22.—Alkaline phosphatase reaction in 7-day graft of anterior lobe of prostate. Great discontinuity and decreased intensity of reaction. × 300.

FIG. 23.—Alkaline phosphatase reaction in graft of anterior lobe of mouse prostate which has been treated with methylcholanthrene. Squamous metaplasia. Only slight reaction remains. × 300.

FIG. 24.—Alkaline phosphatase reaction in graft of anterior lobe of mouse prostate which has been treated with methylcholanthrene. Squamous-celled carcinoma. Complete absence of reaction. × 300.



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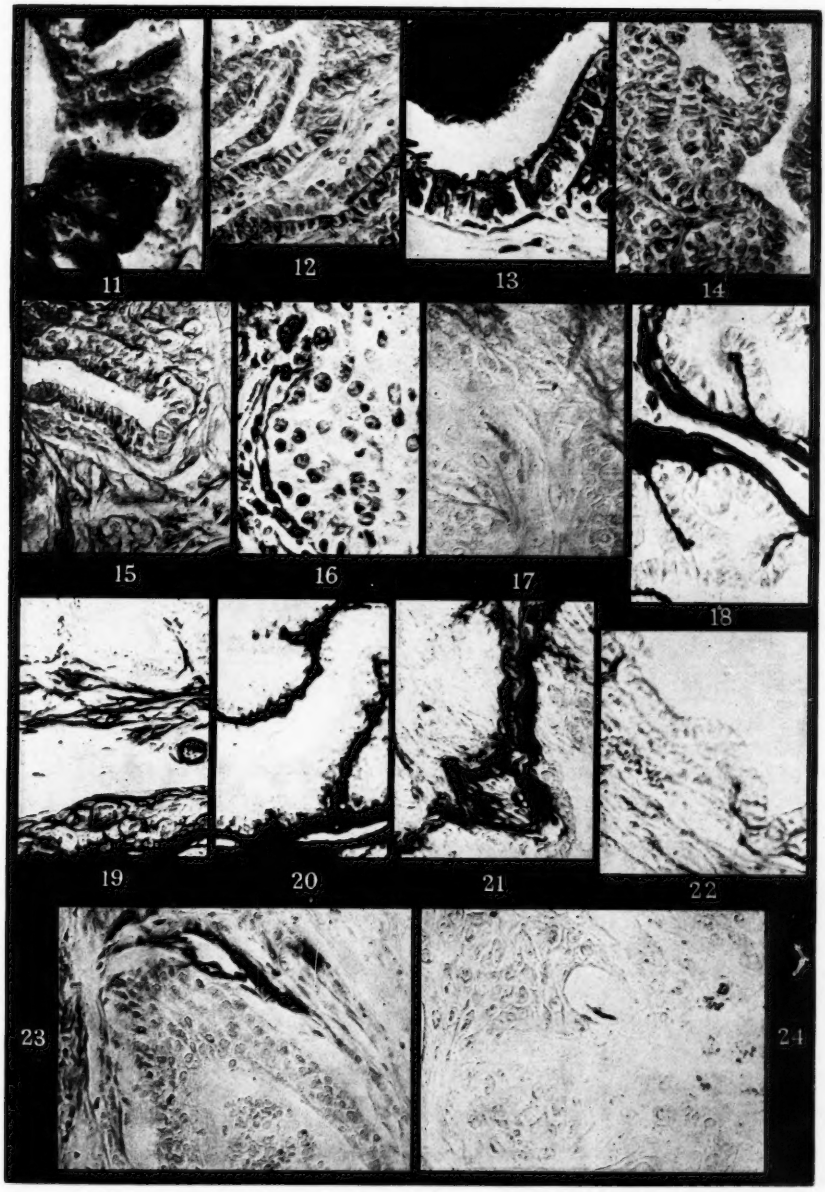

Brandes and Bourne.

and in the nuclei and cytoplasm of human prostatic epithelium by Wolf, Kabat and Newman (1949). Our finding of the remarkable correspondence between the Golgi preparations and the acid phosphatase preparations is new as far as the prostate, and particularly the mouse prostate, is concerned. Phosphatase has, however, been recorded in the Golgi material of other organs. Alkaline phosphatase has been found in the Golgi region of gut epithelium by Bourne (1943), Emmel (1945), Deane and Dempsey (1945), and in the cells of a human papilloma by Manheimer and Seligman (1948). Acid phosphatase has been observed in the Golgi region of the intestinal epithelium, convoluted tubules of the kidney, and uterine epithelium of pregnant rats and sows, and in the seminal vesicle of the mouse by Deane and Dempsey (1945). In view of the changes we found after castration, administration of oestrogens or androgens, in the acid phosphatase reaction in nuclei and in the Golgi region we feel these represent real distributions of the enzyme. In the liver, however, Palade (1951) showed that centrifugally isolated nuclei from homogenates had only 5 per cent of the acid phosphatase activity of the cell.

The significance of the localization of acid phosphatase in the Golgi region is not known, but it may be noted in this connection that the Golgi apparatus has a large phospholipid component; the phospholipid content of seminal fluid is dependent on hormonal stimulation and phospholipids in the ventral prostate of the rat were decreased after castration but restored by the administration of testosterone (Nyden and Williams-Ashman, quoted by Roberts and Szego, 1953). In the human prostate, phospholipids constitute more than 50 per cent of the total lipids of the gland (Scott, 1945). If, as has been suggested in the past, the Golgi region is concerned with phospholipid synthesis, then possibly acid phosphatase is concerned with this process.

Leblond (1950), applying the P.A.S. reaction for polysaccharides to the rat prostate, found positive granules in the Golgi regions of the epithelium. In the mouse we found the same reaction in the Golgi region, and also in the basement membranes and in the sheaths of smooth muscle. As regards the hormonal control of genital phosphatase, Bern (1952) found biochemically an almost complete disappearance of alkaline phosphatase from the rat ventral prostate 120 days after castration without, however, finding changes, either biochemically or histochemically, on the 32nd day. Our results showed that 22 days after castration the alkaline phosphatase reaction had decreased moderately in the basement membrane, and was present mainly in the sheaths of the hyperplastic fibromuscular stroma. We used only 2 hours' incubation, compared with 16 hours in the work quoted and this may account for the difference. Furthermore, we have considered the reduction of intensity in the basement membrane while a positive reaction develops in the muscle sheaths of the hypertrophic fibromuscular stroma. This increase in amount of stroma containing alkaline phosphatase may account for the biochemical finding that the total amount of this enzyme is the same or increased after castration, although it is greatly decreased in its normal sites (basement membranes). Some decrease was seen even 8 days after castration (see also Atkinson, 1948), and Soulairac and Thibault (1948 *a*, *b*) described the disappearance of all genital tract alkaline phosphatase following castration. Meyer and McShan (1950) found biochemically a similar decrease of acid phosphatase, alkaline phosphatase and succinic dehydrogenase in the rat prostate 8 days after castration.

Under the influence of castration or the administration of oestrogens the acid phosphatase of the Golgi region showed changes similar to those described for the Golgi apparatus of the rat and mouse prostates (Moore *et al.*, 1930; Horning, 1947) in similar conditions. In our preparations 4 days after castration the acid phosphatase reaction in the Golgi region showed changes, while the Golgi apparatus still appeared morphologically intact. As Rudolph and Samuels (1949) showed, chemical and metabolic, as opposed to morphological, changes take place rapidly in response to hormones.

Castration also affects enzymes of other organs such as kidney and liver (Kochakian, Garber and Bartlett, 1948; Kochakian and Stettner, 1948). Furthermore, Gersh (1949) found in the Golgi region of duodenal cells a P.A.S.-positive carbohydrate-protein complex which he considered might be arranged in an orderly framework, where diverse enzymes, such as acid and alkaline phosphatase, might be located. In the Golgi region we found a decrease in the P.A.S. material due to castration or oestrogens, while the acid phosphatase lost its compactness, showing mitochondria-like forms, and suggesting that the protein-carbohydrate complex of Gersh binds together mitochondrial elements rich in acid phosphatase. The occurrence of acid phosphatase in mitochondria was shown by Palade (1951).

On the whole, there was great similarity in the effects of castration or oestrogens on the histochemical pattern. Some differences occurred, however, in the basement membrane, where both alkaline phosphatase and P.A.S.-positive material were observed. In castrated mice both decreased, while in the oestrogen-treated animals only the alkaline phosphatase decreased while the P.A.S. reaction became stronger. This also occurred in the grafts treated with 20-methylcholanthrene and this seems to accord with the well known similarity of effects between carcinogenic hydrocarbons and oestrogenic substances (Burrows and Kennaway, 1934; Horning and Dmochowsky, 1947).

All our grafts impregnated with 20-methylcholanthrene rapidly underwent squamous metaplasia (sometimes within 10 days of transplantation) with disappearance of the normal histochemical reactions, while in untreated grafts, after an initial loss of secretory capacity and enzymic patterns, there was a restoration of the normal condition after about 3 weeks. As the stratification and squamous metaplasia proceeded, the epithelial cells seemed to lose their secretory capacity. The P.A.S.-positive secretion and cytoplasmic granules disappeared (probably indicating a shift from glycoprotein to scleroprotein production); the alkaline phosphatase decreased in the basement membrane and the acid phosphatase disappeared from nuclei and cytoplasm, in particular the Golgi region.

Several authors obtained malignant tumours of the prostate by local application of carcinogenic substances to the organ *in situ* (Moore and Melchiona, 1937; Dunning, Curtis and Segaloff, 1946). Horning (1946, 1952), by impregnating mouse prostatic homografts with a carcinogen, obtained squamous cell and glandular carcinomas. This last method was used also for other tissues, such as uterine epithelium (Pan and Gardner, 1948) and bladder mucosa (Hughes, 1949) with the production of malignant tumours. Squamous cell carcinomas developed in some of our grafts impregnated with 20-methylcholanthrene. Once the stage of carcinoma was reached little alkaline or acid phosphatase was left, the latter being present mainly in the kerato-hyalin granules. Acid phosphatase had almost disappeared in these experimental carcinomas whereas it is very abundant in the normal human prostate and in carcinomas of that organ (Gutman, Sproul and

Gutman, 1936; Gutman and Gutman, 1938; Fergusson, 1946). This seems to be due to the fact that most human carcinomas of the prostate are of the glandular type, while the mouse tumours were produced by 20-methylcholanthrene which possesses oestrogenic properties (Burrows and Kennaway, 1934). Oestrogens have been shown elsewhere in this paper to affect the content of both the acid and alkaline phosphatase of the prostate.

The anterior lobe grafts grew better than those of the ventral lobe. Each lobe seems to have a different androgen threshold and to react differently to androgen withdrawal, the anterior lobe being less dependent on this hormone (Horning and Dmochowsky, 1947). In the ventral lobe involution occurs 5 days after castration but not until the 10th day in the anterior lobe (Moore *et al.*, 1930). Presumably in our grafts during the first phase of transplantation and until the establishment of a proper blood supply the amount of androgen reaching the grafts was rather limited; so that the ventral lobes, being more dependent on androgen, were impaired in their growth during this initial phase in comparison with the anterior lobes.

SUMMARY.

A study has been made of the Golgi apparatus, and of the distribution of the histochemical reactions for polysaccharides, acid and alkaline phosphatase, succinic dehydrogenase, and xanthine oxidase in the normal mouse prostate. Acid phosphatase was found to be localized mainly in the Golgi region of the epithelial cells, alkaline phosphatase in the stroma and basement membranes and succinic dehydrogenase in granules in the epithelial cells where also most of the xanthine oxidase was found. The last was in the form of a blue granular reaction and a red diffuse reaction. P.A.S.-reactive substances (polysaccharides) were present in the stroma and basement membranes, in the cytoplasmic granules and in the secretion within the lumen of the acini. Little difference could be seen in the reactions given by the three lobes.

After castration there was fragmentation of the Golgi apparatus, and reduction of the acid and alkaline phosphatase and the P.A.S. reactions. All these reactions reverted to normal after subcutaneous implantation of testosterone.

Implantation of di-ethylstilboestrol produced a similar decrease in the histochemical reactions but even 12 days after removal of the implant they had not become normal.

Subcutaneous grafts of prostatic tissue also showed loss of histochemical reactions, which reappeared when the graft became established.

Grafts of prostatic tissue impregnated with methylcholanthrene showed metaplasia and, in some cases, squamous cell carcinoma developed.

In both metaplasia and carcinoma there was an almost complete loss of normal histochemical reactions.

We are indebted to Dr. J. Craigie for having kindly supplied us with the mice used in this work.

The stilboestrol and testosterone implants were kindly supplied by Organon Laboratories Ltd.

The expenses of this research were partly defrayed out of a block grant from the British Empire Cancer Campaign.

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THE STABILITY OF A RECOMBINANT INFLUENZA VIRUS.

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Received for publication August 17, 1954.

IN the earliest work on recombination between strains of influenza virus, a mixture of two antigenically distinguishable influenza A strains was inoculated intracerebrally into mice. Suspensions of infected mouse brains were then inoculated into eggs, from which were isolated new types of virus exhibiting properties of both parent strains. The most striking effect observed was the possession of neurotropism by some derivative strains which were serologically indistinguishable from the non-neurotropic parent virus (Burnet and Lind, 1951*a*, *b*). A detailed study was made by Burnet and Edney (1951) of the virus types produced after inoculation of a mixture of MEL—a non-neurotropic influenza A strain—and NWS—the neurotropic variant of the classical WS A strain. Recombinants were obtained which were serologically MEL and exhibited different degrees of neurotropism. Appleby (1952) also isolated a new neurotropic strain, neuro-Kunz, by inoculation of a mixture of two ultra-violet irradiated strains, NWS and Kunz.

More recently, inoculation of a mixture of two viruses into eggs has shown that as well as types with the serological character of either parent, viruses may be obtained which combine antigenic qualities of both parents (Hirst and Gotlieb, 1953*a*, *b*; Fraser, 1953; Burnet and Lind, 1953; Gotlieb and Hirst, 1954). Some of these combination forms could not be serially propagated as such in eggs, but readily reverted to the parent types. This led to criticism of the earlier claim that the association of neurotropism with the serological characteristics of a non-neurotropic strain represented the production of a new recombinant virus, mainly on the ground that this association had not been shown to be stable through a series of egg passages. It was suggested that the neurotropism, demonstrable only by mouse inoculation, might be due to the persistence of NWS as a contaminant (Hirst and Gotlieb, 1953*a*).

We report here an investigation of the stability of the properties of the strain neuro-MEL on repeated passage in eggs, showing that the strain is a single virus entity whatever may be the mechanism of its production.

MATERIALS AND METHODS.

Virus strains.

Three strains of influenza A were used, MEL (Burnet, 1935), NWS (Stuart-Harris, 1939) and neuro-MEL (Burnet and Edney, 1951). The neuro-MEL was isolated from mouse brain after intracerebral inoculation of a mixture of MEL and NWS. After the virus had been passaged three times at high dilution in eggs, infected allantoic fluids were freeze-dried. No special precautions of storage were taken and the freeze-dried stock was revived 22 months later. After two passages in eggs, a seed virus was stored in a dry-ice cabinet.

Virus stocks were prepared by allantoic inoculation of 11-day chick embryos with 0·2 ml. volumes of seed virus diluted 10^{-5} in broth. After incubation at 35° for 42 hr., the eggs were chilled at 4° and fluids harvested and stored at 4°.

Haemagglutination titrations.

Haemagglutination titrations were carried out by the pattern method in plastic trays. Virus was diluted in twofold steps in 0·25 ml. volumes of normal saline, an equal volume of 1 per cent fowl red cells added and the pattern read after 40 min. at room temperature. The smallest amount of virus giving partial agglutination was taken as one agglutinating dose (A.D.). The haemagglutination (HA) titre was the reciprocal of the initial dilution of virus at this end-point.

Haemagglutination-inhibition titrations.

Serial twofold dilutions of serum were prepared in normal saline in 0·25 ml. volumes. To each cup 0·25 ml. of 1 per cent fowl red cells and one drop of virus containing 5 A.D. were added. After 40 min. at room temperature the pattern was read, the HA-inhibition titre of the serum being taken as the reciprocal of the highest dilution of serum giving partial agglutination.

Inhibition titrations for determination of indicator state of viruses.

A preparation of 10 per cent egg-white in normal saline was used as the standard inhibitor. Serial twofold dilutions of inhibitor were prepared in 0·25 ml. volumes in normal saline and one drop of virus containing 5 A.D. added to each dilution. After 30 min. at room temperature 0·25 ml. of 1 per cent fowl red cells was added to each cup and the pattern read after 40 min. The inhibitory titre was taken as the reciprocal of the highest dilution of inhibitor showing partial agglutination.

Preparation of antisera.

Antisera were prepared by inoculating rabbits intravenously with a small dose of virus (1 ml. of undiluted allantoic fluid—approximately 1300 A.D.), and 24 days later a heavy dose of centrifuged concentrated virus (80,000 A.D.). The rabbits were bled at 6-day intervals after the second dose and the sera with the highest titres chosen. Usually the peak of antibody production was attained about 30 days after the first inoculation.

Sera were absorbed by a method similar to that described by Hirst and Gotlieb (1953*a*). They were treated with RDE (Burnet and Stone, 1947) for 2 hr. at 37°. Heterologous virus was concentrated 10 times by centrifugation and added to the serum-RDE mixture to give a final dilution of 1/9 of serum. The mixture was left overnight at 4° and centrifuged at 29,000 **g.** for 1 hr. The supernatant fluid was heated at 65° for 30 min. in the presence of 2 per cent citrate to inactivate RDE and any residual virus, the final dilution of the serum being 1/10. It was found by haemagglutination-inhibition tests that this treatment reduced heterologous antibody titre to less than 20, while leaving the homologous antibody virtually intact.

Neutralisation titration in mouse brain.

The infectivity titres of stocks of NWS and neuro-MEL stored in dry-ice were estimated by intracerebral inoculation of 0·03 ml. volumes of 10-fold dilutions of virus into groups of 4 mice. Deaths up to 14 days were recorded and the LD_{50} estimated by Thompson's moving average method (Thompson, 1947). Fivefold dilutions of serum in 0·25 ml. volumes were mixed with 0·25 ml. of virus, diluted to contain 10,000 LD_{50}. The same amount of virus was mixed with broth-saline, and with normal rabbit serum diluted 1/5, as controls. Of each mixture 0·03 ml. was inoculated intracerebrally into groups of 3 mice, and deaths up to 14 days were recorded.

Mouse pathogenicity tests.

Fresh infected allantoic fluids or fluids which had been stored in dry-ice were diluted 10^{-3} in broth saline and 0·03 ml. inoculated intracerebrally into 3 mice. Mice were examined daily for 14 days by twirling by the tail, and symptoms and deaths recorded. A virus was considered to be pathogenic if all mice in the group exhibited clonic convulsions or died.

Electron microscopy.

Virus was adsorbed to fowl red cell ghosts as described by Dawson and Elford (1949). A Siemens electron microscope was used with a 50 μ objective aperture and an accelerating voltage of 50 kV.

RESULTS.

Determination of Marker Characteristics of the Viruses.

The behaviour of NWS, MEL and neuro-MEL strains in various *in vivo* and *in vitro* tests has been described previously (Burnet and Lind, 1951*b* ; Burnet and Edney, 1951). There are four especially useful marker characteristics, namely, serological type, heat stability of haemagglutinin, indicator state of the virus after heating and mouse pathogenicity. The properties of the NWS, MEL and neuro-MEL in respect of these marker characteristics were checked many times. It was found that the haemagglutinins of MEL and neuro-MEL were relatively stable to heating at 56° for 30 minutes, that of NWS labile. Neuro-MEL was readily inhibited by mucoid inhibitors after heating ; MEL required the addition of citrate before it became an indicator on heating (Stone, 1949). NWS could not be tested for indicator state owing to the lability of its haemagglutinin. Neuro-MEL and NWS were both pathogenic to mice when inoculated intracerebrally ; MEL was not. It was established by haemagglutination-inhibition tests that neuro-MEL was a virus of the MEL serological type. These results fully confirmed those of the previous workers.

Neutralisation of Neuro-MEL and NWS in the Mouse Brain.

The test for marker characteristics ensured that the viruses used were predominantly of the desired types but could not reveal the presence of small amounts of a contaminant virus. For this purpose a mouse neutralisation test was used. Since both MEL and NWS are type A strains there exists a low level of serological cross-reactivity between them. Hence for cross-neutralisation experiments absorbed sera were essential. The results of haemagglutination-inhibition titrations of the unabsorbed and absorbed sera are shown in Table I.

TABLE I.—*Haemagglutination-Inhibition Titrations.*

	MEL antiserum.		NWS antiserum.	
Virus.	Original.	Absorbed with NWS.	Original.	Absorbed with MEL.
NWS	670	<20	5040	3780
MEL	5300	3400	200	<20
Neuro-MEL	5300	3400	340	<20

The figures represent the HA-inhibition titres of serum tested against 5 A.D. of virus.

The absorbed sera were used for cross-neutralisation tests in mouse brain in which the MEL and NWS antisera were tested against the two neurotropic strains, neuro-MEL and NWS. Table II gives the results.

MEL and neuro-MEL were indistinguishable in the haemagglutination-inhibition test, but readily distinguishable from NWS. In the intracerebral mouse pathogenicity test neuro-MEL was neutralised by MEL antiserum and not by NWS antiserum. The neurotropism of neuro-MEL was therefore concluded

TABLE II.—*Cross-neutralisation Test of Neuro-MEL and NWS in the Mouse Brain.*

Virus.	Dilution of MEL antiserum. 1/50.	1/250.	1/1250.	1/6250.	Dilution of NWS antiserum. 1/50.	1/250.	1/1250.	1/6250.	Controls. Broth saline.	Normal serum 1/5.
NWS	3/3	3/3	3/3	3/3	0/3	0/3	0/3	3/3	3/3	3/3
Neuro-MEL	0/3	0/3	0/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3

Numerator : number of mice which died within 14 days of inoculation.
Denominator : number of mice inoculated.

to be due to a virus of MEL serological character, and not to NWS present either as a contaminant in the original fluid or produced by reversion of neuro-MEL on passage.

Serial Egg Passage of Neuro-MEL.

The stability of the neuro-MEL strain was tested by serial passage in eggs. Tenfold dilutions of the seed virus were made in broth and inoculated into groups of 11-day-old chick embryos. After 3 days' incubation, fluids were harvested and tested by a rapid haemagglutination spot test. Those found to be infected were stored at 4° except for a small portion which was ampouled and stored in dry-ice. One fluid from the group of eggs infected with the highest dilution of inoculum was used for passage by the same technique. Four such passes were made. All fluids positive at the highest dilution in each pass and some from lower dilutions were tested for serological type, heat stability of haemagglutinin and indicator state after heating. Controls of MEL and NWS were always included. Selected fluids were also tested for mouse pathogenicity. Table III summarises the results of these tests. In no case was there evidence of double neutralisation or of the presence of either parent type.

TABLE III.—*Serial Egg Passage of Neuro-MEL at High Dilutions.*

Dilution.	Pass 1.				Pass 2.						Pass 3.				Pass 4.			
10^{-9}	–	–	–	–	–	–	–	–	..	..	–	–	–	–	*NM*	*NM*	–	–
10^{-8}	–	–	–	–	*NM**	*NM*	*NM*	NM	NM	NM	*NM**	*NM*	–	–	NM	NM	–	..
10^{-7}	*NM**	*NM*	*NM*	–	NM	+	+	+	+	+	NM	NM	+	+	NM	+	+	+
10^{-6}	+	+	+	..	NM	+	+	..	..	..	NM	NM	+	+	NM	+	+	+
10^{-5}	NM	+	+	..		..	..	..	..	..	..	..	..	..				

– = no HA detectable after 3 days' incubation.
\+ = positive HA at 3 days : not tested further.
NM = identified as neuro-MEL by three *in vitro* tests.
NM = also tested in mice and found to be pathogenic.
* = used for subsequent pass.

One fluid from the third pass was inoculated at " limiting " dilution ($10^{-8.8}$) into 20 eggs. Small amounts of allantoic fluid were removed from each egg on the 2nd day after inoculation and tested for haemagglutinin. Positive eggs were chilled and harvested. Sampling, testing and harvesting were continued in this way on the 3rd, 4th and 5th days. Eventually, 6/20 eggs were found to be infected. The results of the various tests on the 6 virus fluids are shown in Table IV. There is no indication whatever of the occurrence of reversion to parent type during growth within the egg or of the presence of a contaminating virus.

TABLE IV.—*Stability of Recombinant Neuro-MEL on Passage Virus at Limiting Dilution.*

Virus.		HA titre.		HA-inhibition titre.		Inhibitory titre of egg white.			Mouse pathogenicity.
		Living virus.	Heated virus.	MEL antiserum.	NWS antiserum.	Living virus.	Heated virus.	Virus heated in citrate.	
Neuro-MEL fluids	1	480	240	3000	<500	<100	2000	2000	+
	2*	13	<10	..	..	..	..	..	..
	3	480	100	3000	<500	..	1700	..	+
	4	120	30	3000	<500	..	1000	1200	+
	5	80	60	3000	<500	..	1000	..	+
	6	960	120	3000	<500	..	1100	..	+
MEL		960	320	3000	<500	<100	<100	2000	−
NWS		140	<10	<500	4000	..	..	..	+

* This fluid was re-passed in eggs and found to behave like neuro-MEL.

Electron Microscopy.

It was of interest to see whether the recombinant strain neuro-MEL differed in morphology from the parent strains. Electron micrographs of each virus adsorbed to fowl red cell ghosts were indistinguishable. In each case the virus was chiefly in the form of discrete spherical particles, but a few short rods were present.

DISCUSSION.

The production of viruses having some of the marker characteristics of each of two parent strains is usually attributed to the recombination of parental sub-units during the process of virus replication within the host cells. This is not the only possible interpretation. Other mechanisms more analogous to the transformation of pneumococcal types have been suggested and some sceptics would interpret the facts on the basis of simple contamination of the derivative virus with one of the parent strains. The work reported here does not attempt to analyse the nature of the mechanism involved but, by establishing the purity of one such virus, refutes the contamination hypothesis.

The neuro-MEL virus showed a lower grade of virulence for mice on intracerebral inoculation than did the NWS virus. There was also some variation of its virulence during serial egg passages. A quantitative study of this variability was not attempted, but within the limited scope of the experiments there was no indication of either progressive exaltation or progressive attenuation of its neurotropism.

SUMMARY.

The recombinant neuro-MEL obtained by inoculation into mouse brain of a mixture of MEL and NWS was tested for purity and stability of its properties during egg passage.

The possibility of its neurotropism being due to small amounts of the parent NWS virus present as a contaminant was excluded by cross neutralisation tests in mouse brain.

The neuro-MEL strain retained its marker characteristics unchanged through four serial egg passages at high dilution.

Of 20 eggs inoculated with egg passage virus at limiting dilution, 6 became infected ; these all yielded typical neuro-MEL virus and gave no evidence of the occurrence of contamination or of reversion to parent type.

We are indebted to Professor Wilson Smith, F.R.S. for his advice and guidance. The electron microscopy was done by Dr. Heather B. Donald of the National Institute for Medical Research. The work was supported by personal and expense grants from the Medical Research Council.

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OBSERVATIONS ON THE GROWTH OF HERPES SIMPLEX VIRUS IN THE CHORIO-ALLANTOIS OF THE DEVELOPING CHICK EMBRYO AND IN TISSUE CULTURE.

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Received for publication August 26, 1954.

ALTHOUGH the cycle of growth of virus in susceptible host cells has been investigated most intensively with bacterial and influenza viruses, its study with those viruses multiplying in the ectodermal cells of the chorio-allantoic membrane (C.A.M.) of the developing chick embryo (D.C.E.) has been limited to vaccinia (Briody and Stannard, 1951), lymphocytic choriomeningitis (Tobin, 1954) and herpes simplex virus.

The growth of herpes simplex virus in this site has been studied by Shaffer and Enders (1939) and a paper by Scott, Coriell, Blank and Grey (1953) has been published since our work was completed. Herein, further observations on the growth of this virus and the production of complement-fixing antigen in the C.A.M. have been made, together with a comparison of its growth in tissue culture of the Maitland type.

MATERIALS AND METHODS.

Two strains of virus were used in this study : (1) EWH, kindly supplied by Dr. E. Weston Hurst who isolated it from the brain of a case of encephalitis. When received it had not been passed in chick embryos but before this study commenced it was passaged 10 times chorio-allantoically ; (2) McC strain, kindly supplied by Dr. K. McCarthy, which had been passed for some years in the C.A.M. of the D.C.E. Most experiments reported here were done with the EWH strain.

Technique of inoculation and harvesting of C.A.M.

The chorio-allantois of 12–13-day-old chick embryos was inoculated by the method of Beveridge and Burnet (1946) with 0·05–0·1 ml. of virus suspension and then incubated at 37° until harvested. This was done by cutting through the shell, outside the reflection of the inoculated portion of the C.A.M., both being removed intact together with any fluid on the ectodermal surface of the membrane. In this way, virus which had not been absorbed by the membrane could be collected in the inverted piece of shell. Each C.A.M. was washed in 1 ml. of Hartley broth and weighed before being transferred to a mortar for grinding with small glass beads and a variable measured amount of Hartley broth (less than 1 ml. per membrane). After grinding, the material was shaken mechanically in a wide mouth test tube for 2–3 min. The suspension was then allowed to stand at 4° for 1 hr. and the supernatant removed and titrated for infectivity by the pock-counting method of Beveridge and Burnet (1946) using 4–6 eggs per 10-fold dilution. Seed virus was prepared from the C.A.M. in a similar manner, but in addition the material was centrifuged at 5000 r.p.m. in a wide angle centrifuge for 10 min. and the supernatant removed and stored at − 35° until required for inoculation. Unabsorbed virus was calculated by titrating the C.A.M. washings combined with the fluid obtained from the excised piece of shell.

The C.A.M. extracts from the growth experiments, if required for complement fixation tests, were centrifuged at 2500 r.p.m. for 15 min. in a horizontal centrifuge. Dilutions of

the supernatant of this spinning were tested with human anti-herpetic serum, 2 M.H.D. of complement and fixation for 18 hr. at 4°. The haemolytic system consisted of 2 per cent sheep cells sensitized with 5 M.H.D. of haemolysin ; the test was read after 30 min. in the water-bath at 37°.

Tissue culture technique.

Cultures of the type introduced by Maitland and Maitland (1928) were used, in which herpes simplex virus has been shown to grow well (Thompson and Coates, 1942). Approximately 0·25 g. of chick embryo cut into small fragments was added to 50-ml. Erlenmeyer flasks containing 3·5 ml. of a mixture of Tyrode's solution, 68 parts ; rabbit serum, 28 parts ; and viral suspension in Tyrode's solution, 4 parts. Penicillin and streptomycin were added to give a concentration of 400 units and μ g. respectively per ml. After gentle shaking at 22° for 10–15 min. the flasks were placed in an air-tight tin containing a small reservoir of water and incubated at 37°. The contents of the flasks were removed as required and the tissue fragments ground in a mortar either with the fluid phase or after they had been separated by centrifugation and washed once in Tyrode's solution. The washings and the supernatant were combined to determine the virus titre of the fluid phase of the culture. The virus content of the tissue phase was calculated from the supernatant obtained, after grinding the fragments in a measured amount of Hartley broth, by allowing the suspension to stand for 1–1½ hr. at 4°. Virus titres were expressed as the number of pocks per membrane or ml. of fluid.

RESULTS.

Growth of virus in the C.A.M.

To determine the general nature of the growth of herpes simplex virus over a period of days, 12–13-day embryos were inoculated by the chorio-allantoic route and incubated at 36–37°. Groups of 4 eggs were harvested and titrated for virus at different time intervals from 1 to 120 hr., the results of sample experiments being recorded in Table I. Following inoculation with different doses of virus there was a rapid reduction in the amount of infective virus present in the C.A.M., only 1 to 10 per cent of the inoculum being demonstrable during the first few hours. A small increase in virus occurred between 1 and 10 hr., but after that the amount of infective virus increased rapidly to its maximum at 48 hr., and then slowly diminished in quantity during the following 3 days.

TABLE I.—*The Increase in Infective Virus and in Complement-fixing Antigen after Inoculation of the C.A.M. with Herpes Simplex Virus.*

Hours after inoculation.	Inoculum (pocks per C.A.M.) (1) $5·6 \times 10^2$ T/I	C.F.T.	(2) $1·2 \times 10^3$ T/I	C.F.T.	(3) $6·5 \times 10^4$ T/I	C.F.T.	(4) $6·0 \times 10^5$ T/I	C.F.T.	(5) $2·1 \times 10^7$ T/I
1	0·01	< 4	0·07	< 4	0·04	< 4	0·09	< 4	0·08
3	0·02	< 4	0·14	< 4	0·05	< 4	—	—	0·07
6	0·05	< 4	0·21	< 4	0·09	< 4	0·08	< 4	0·05
10	0·10	< 4	0·80	< 4	0·13	< 4	0·05	< 4	0·13
24	9·6	8	420	20	42	16	16	32	170
48	450	30	13000	30	410	32	—	—	—
72	400	40	2800	40	110	48	—	—	—
96	250	30	830	40	46	32	—	—	—
120	140	30	600	30	15	16	—	—	—

T/I.—Ratio of titre of virus in the C.A.M. to titre of the inoculum.

C.F.T.—Complement-fixing titre given as reciprocal of the highest dilution giving complete fixation.

In experiments 1–3 virus used as inoculum was harvested at 48 hr.; in experiments 4–5 it was harvested at 18 hr.

Concentrations of infective virus during the initial stage and during the first 12 hr. of rapid growth were then studied in more detail.

The amount of virus obtained from the membranes and from their washings during the first 10 hr. of growth were determined at different intervals of time. The results in Table II show that by 15 min. more than 84 per cent of the virus inoculated had been removed by the C.A.M. and of this at least 93 per cent had lost its infectivity. After the initial rapid absorption the amount of virus found in the C.A.M. and in the washings remained either relatively constant or else increased somewhat in the membrane as had been noted in the previous experiments.

TABLE II.—*Distribution of Infective Herpes Simplex Virus in the Chorio-allantoic Membrane and Surface Washings during the First Ten Hours of the Growth Cycle.*

	Size of inoculum (pocks per membrane)					
	460		11,200		3,500	
Time after inoculation.	Washings.	C.A.M.	Washings.	C.A.M.	Washings.	C.A.M.
0 min.	315*	0	—	—	3600	40
5 ,,	110	0	—	—	400	160
10 ,,	175	0	—	—	—	—
15 ,,	50	15	254	180	370	200
20 ,,	—	—	—	—	—	—
30 ,,	70	15	292	300	360	80
40 ,,	—	—	—	—	260	100
60 ,,	—	60	184	83	240	160
2 hr.	—	—	—	—	280	240
3 ,,	—	20	—	—	450	560
4 ,,	—	60	—	—	—	—
5 ,,	—	150	—	200	180	—
6 ,,	—	120	—	—	—	—
7 ,,	—	—	—	—	160	480
10 ,,	150	520	630	500	120	880

* Figures expressed as average number of pocks in washings or per membrane.

As the greatest increase of virus occurred between 10 and 24 hr., this part of the phase of the growth cycle was investigated in greater detail. Groups of four eggs were harvested at approximately hourly intervals and the virus content of the C.A.M. titrated. The results are given in Table III. By 13–14 hr., the amount of virus found in each membrane approximated to the quantity inoculated and between then and 24 hr. increased from 160–640-fold. In each case there was some indication that the increase was not even, but occurred in a series of steps at intervals of 4–6 hr., each step increasing the titre of virus by approximately 10-fold.

In the foregoing experiments, the virus used as inoculum had been harvested from eggs incubated for 48 hr. at which time virus had stopped increasing rapidly. To find out if virus taken from the phase of rapid increase behaved differently, two growth curves were obtained using virus harvested between 18 and 20 hr. after inoculation and re-inoculated into fresh eggs within 30 min. The results which are also given in Table I (columns 4 and 5) show that virus from this phase behaved in a similar manner to that harvested during the stationary one, except that the slow increase in infective virus during the early hours after inoculation did not occur.

Development of complement-fixing antigen during virus growth in the C.A.M.

No complement-fixing antigen was found in the C.A.M. during the first 10 hr. of growth when inocula from $5{\cdot}6 \times 10^2$ to 6×10^5 were used (Table I); by 24 hr. it was demonstrable and after rising to a maximum at 72 hr., slowly decreased. To determine when complement-fixing material first could be detected, tests were done on the 15–25-hr. harvestings (see Table III). It appeared about 14 hr. after inoculation and rose slowly. There was no correlation between the titre of infective virus and amount of complement-fixing antigen. In order to exclude non-specific fixation of complement, a herpes-negative serum which reacted non-specifically with normal C.A.M. was also used to see if in infected membranes more material which would fix complement non-specifically was found than in normal membranes. Although this did seem to be so, the rise was very slight, being no more than one doubling dilution of antigen. The fixation of complement from 14 hr. onward would thus seem to be specific.

TABLE III.—*Growth of Herpes Simplex Virus* 10–25 *hours after C.A. Inoculation.*

Hours after inoculation.	Inoculum. $2{\cdot}6 \times 10^3$ T/I	$2{\cdot}6 \times 10^3$ C.F.T.	$3{\cdot}8 \times 10^3$ T/I	$3{\cdot}8 \times 10^3$ C.F.T.	$7{\cdot}4 \times 10^3$ T/I	$7{\cdot}4 \times 10^3$ C.F.T.
10	—	—	—	—	0·5	< 4
11	0·1	< 4	0·1	< 4	—	—
12	0·2	< 4	0·3	< 4	0·6	< 4
13	0·3	< 4	1·0	< 4	2·4	< 4
→						
14	3·2	4	1·2	< 4	3·5	4
15	—	—	→ —	—	→ —	—
16	3·6	4	10	4	35	4
17	4·2	4	13	4	35	4
→						
18	37	4	—	—	68	4
19	50	4	18	4	99	4
					→	
20	—	—	—	—	380	4
21	57	8	18	8	—	—
22	→ —	—	21	8	430	8
23	120	8	32	8	400	8
			→			
24	690	12	140	8	460	16
25	—	—	160	12	—	—

T/I—Ratio of virus titre to inoculum.
C.F.T.—Reciprocal of antigen dilution giving complete fixation.
→ Step-like increase of virus.

Growth of virus in tissue culture.

After inoculation of groups of flasks with different doses of virus, one flask from each series was harvested at different time intervals and the contents titrated for infective virus. As with growth in the C.A.M., the amount of virus obtainable during the first phase of the growth cycle was only a small fraction of that inoculated. Increase in virus to a level exceeding that inoculated was sometimes delayed up to 24 hr. and its maximum concentration was not reached until 96 hr., after which it decreased rapidly in amount (Table IV).

TABLE IV.—*Growth of Herpes Simplex Virus in Tissue Cultures of the Maitland Type.*

Hours after inoculation.	Inoculum (pocks per flask). $1·0 \times 10^3$ T/I	$1·5 \times 10^3$ T/I	$1·8 \times 10^3$ T/I	$1·6 \times 10^5$ T/I
2	—	—	—	0·01
4	—	—	—	0·03
6	—	—	—	0·05
8	0·1	0·07	0·20	0·19
18	—	0·27	0·78	—
24	0·96	0·80	3·0	0·19
48	14	22	78	0·90
72	130	2000	870	15
96	1400	6700	2100	6·3
120	240	530	370	0·7
144	—	—	90	—

T/I—Ratio of virus titre per flask to inoculum.

Titration of both the fluid and tissue phases separately showed (Table V) that over 90 per cent of the inoculum had disappeared within 30–60 min. and that when infective virus began to increase, the greater proportion of it was in the fluid phase, that in the tissue increasing little and remaining at a low level. The earlier part of this increase from the 2nd to the 9th hour after inoculation was more probably due to the release of some of the inoculated virus from the surface of cells unable to absorb it, than to the production of new infective particles.

TABLE V.—*The Concentration of Herpes Simplex Virus (EWH strain) in Fluid and Tissue Phases of Maitland Type Tissue Cultures.*

Hours after inoculation.	Inoculum (pocks per flask). $1·4 \times 10^5$ Fluid phase.	$1·4 \times 10^5$ Tissue phase.	$1·2 \times 10^5$ Fluid phase.	$1·2 \times 10^5$ Tissue phase.
½	—	—	$5·7 \times 10^3$*	$4·4 \times 10^2$
1	$7·2 \times 10^3$	$7·5 \times 10^2$	$7·2 \times 10^3$	$2·2 \times 10^3$
2	$4·0 \times 10^3$	$1·2 \times 10^3$	$2·2 \times 10^4$	$1·0 \times 10^3$
3	—	—	$2·6 \times 10^4$	$1·4 \times 10^3$
4	$1·8 \times 10^4$	$1·4 \times 10^3$	$2·9 \times 10^4$	$1·2 \times 10^3$
5	—	—	$5·1 \times 10^4$	$2·0 \times 10^3$
6	$2·4 \times 10^4$	$2·1 \times 10^3$	—	—
9	$5·4 \times 10^4$	$2·8 \times 10^3$	$5·7 \times 10^4$	$3·1 \times 10^2$
24	$1·4 \times 10^5$	$3·7 \times 10^3$	$3·8 \times 10^5$	$5·2 \times 10^3$

* Total number of pocks produced.

A comparison was made between the behaviour of virus in living tissue culture, first, with virus in a culture containing cells killed by heating in a water-bath for 5 min. at 100°, secondly, with virus suspended in serum-Tyrode solution and thirdly, with virus in the presence of chick embryo extract, prepared by grinding up two chick embryos in 5 ml. of serum-Tyrode mixture and spinning down the coarser particles at 1500 r.p.m. for 10 min. As would be expected virus disappeared rapidly in the living tissue, less than 5 per cent of the inoculum being detectable at 2 hr., while in the heated tissue this fall was somewhat slower. The level in the living culture had risen at 4 hr. and increased steadily thereafter, while with heated cells the virus content declined from the start, being less than 0·2 per cent by 9 hr. and by 24 hr. had disappeared completely. In serum-Tyrode solution

containing tissue extract at 37°, the drop to zero was delayed until after 24 hr., while in serum-Tyrode solution alone at 37° virus could still be detected after 72 hr. with a comparable inoculum. In both these media the decline seemed more rapid initially than later. The presence of intact cells, either alive or dead, to which virus could be adsorbed, accelerated virus disappearance as compared with extracts of the same tissue or with incubation alone. Virus held at 4° in serum-Tyrode solution decreased in titre only slowly as compared with its reduction at 37°, there being no observable fall after 7 days at this temperature.

DISCUSSION.

The strains of herpes simplex virus used in these experiments have been shown to undergo a definite cycle of growth in the chorio-allantois of the developing chick embryo. The general pattern followed that already noted by Shaffer and Enders (1939) and Scott *et al.* (1953) with their strains and is similar to that found with most other virus-host systems investigated. This cycle can be divided into 5 phases: (1) the phase of adsorption of the virus to cells; (2) the eclipse phase in which only a small proportion of the inoculated virus can be detected; (3) the phase of rapid increase of virus; (4) a stationary phase of maximum concentration of infective virus, and (5) the phase of decline.

The majority of infective particles were absorbed within 15 min. by the chorio-allantoic cells and remained there in an undetectable form for the next 10–12 hr. before any marked increase occurred. However, during this time there was a slow increase in infective titre within the cells while that in the surface washings remained fairly constant. Although the majority of virus particles were transformed into a non-infective or vegetative form, a small percentage remained infective. This proportion was fairly constant and could not be explained by assuming it was free unadsorbed virus. If it were it should have been adsorbed at a rate proportional to that of the greater part of the inoculum and have disappeared. Therefore, it was assumed that it was prevented from becoming vegetative virus because it was in contact with cells which could adsorb it but which could not provide the necessary environment for its change to vegetative particles. Perhaps it was held by these cells tightly enough while in the egg but was removed by the mechanical procedures involved in washing and harvesting the C.A.M. That this virus was on the cell surface was supported by the observation of both Shaffer and Enders (1939) and Scott *et al.* (1953) that the majority of this infective virus could be neutralized by adding antiserum to the C.A.M. without altering the number of pocks produced. Intracellular virus should not be inactivated by this treatment. A similar suggestion has been made by Henle (1949) in regard to the adsorption of influenza virus. It would thus seem that although the majority of cells of the chorio-allantois are capable of producing virus, a few are not endowed with this property. Where increase of virus during the earlier part of the latent phase was observed it was probably more apparent than real in the sense of active production of virus and could be explained by the splitting up of small aggregates on the cell surface, the individual particles being released by the disintegration of the chorio-allantois. This increase occurred in each case when the inoculum was virus harvested at 48 hr. but not when it was virus gathered at 18 hr., the individual infective particles perhaps being released more easily from the cell matrix by grinding at 18 hr. than later in the

growth cycle. That intact C.A.M. cells and ultrafiltrates of cells could markedly affect the titre of a virus suspension has been noted by Tobin (1953) and Krueger and Baldwin (1937).

After 10–11 hr. virus tended to increase more rapidly until by 13–14 hr. the number of infective particles had surpassed that of the inoculum. If this point is assumed to be the end of the first cycle the increase of the previous 2–4 hr. would be due either to infectious particles accumulating within the cell or to a few completing this cycle somewhat earlier than their fellows. After this time the concentration remained relatively constant until the next cycle produced another step-like increase of virus 4–6 hr. later. It would appear that the virus was in the vegetative form for the greater part of the first cycle, becoming infective only in the last part. The step-wise increase was consistent with virus in the succeeding cycles going through successive phases of disappearance and reappearance, the plateaux being merely the resultant of the amount of maturing and disappearing virus, the appearance of mature virus in the following cycle again producing a sudden rise in total detectable infectivity.

Fourteen hr. after inoculation complement-fixing antigen appeared in the membranes and slowly rose but showed no correlation with the amount of infective virus present. With the EWH strain in infected amniotic fluid a titre of over 10^7 infective particles/ml. was required before complement fixation could be obtained. However, its appearance in the C.A.M. with virus titres between $8{\cdot}4 \times 10^3$ and $3{\cdot}8 \times 10^4$ would indicate that virus material, other than that in the infective form, was being produced. How early such material was found could not be determined owing to the insensitivity of the herpes simplex complement-fixing system. During increase of virus the ratio of non-infective to infective virus material decreased until the point of maximum infectivity was reached at 48 hr. after which it again started to increase, infectivity falling off more rapidly than complement fixation. Scott *et al.* (1953) also found no complement fixation during the first 12 hr. of growth but did not investigate the interval between 12 and 24 hr. for its appearance.

When virus harvested either during the stationary phase, or in the phase of rapid increase, was used as an inoculum both behaved similarly, indicating that it was the state of metabolism of the cell rather than any property of the virus particles which controlled their growth rate. During the phase of rapid increase, the cells of the chorio-allantois could be seen to be reacting by 15 hr. or so to the presence of virus by hyperplasia and hypertrophy, and later pock formation, at the same time supplying the virus with better conditions for its multiplication; as the cells disintegrated following this hyperplasia, virus multiplication slowed down until destruction of virus overshadowed the production of new infective particles.

In tissue culture, with cells not metabolizing under optimal conditions, virus growth was very much slower and the time required to reach the maximum level of infectivity was double that with the intact C.A.M. The level of infective virus detectable in the cellular phase of the tissue cultures remained relatively constant for more than 24 hr. although virus was being produced as judged by the rise in titre in the fluid phase. A large proportion of the increasing virus must therefore have been in the vegetative form during this multiplication if the marked rise in titre between 9 and 24 hr. was true virus multiplication and not just release of the inoculum from the site of adsorption. It is impossible to decide what proportion

of the inoculum is absorbed on cells capable of supporting virus growth in tissue culture as it is with the C.A.M., where the eventual production of pocks shows the amount of virus passing through multiplication cycles.

From these results, the following tentative picture of the multiplication of herpes simplex virus can be formed which differs somewhat from that suggested by Scott *et al.* (1953). Following inoculation most of the virus is taken up by the host cells within a few minutes and is changed into the vegetative state. A small proportion is attached to cells and is held loosely at the cell surface, where small aggregates tend to be broken up and easily detached by mechanical means. Towards the end of the first full cycle infective virus begins to appear in the host cells and is later released and transferred to the surrounding tissue, which is hyperplastic as a defence mechanism against the invader. These cells offer a better medium for virus multiplication, and the ensuing cycles of reproduction, although similar in type to the first one, take less time to complete, giving at first 10-fold step-like increases of virus until the variations in the length of different virus cycles produce an apparently even increase of virus as each gets more and more out of step with its neighbour. During this phase the proportion of infective to non-infective material increases as relatively more active virus is produced from the resources available. As cellular hyperplasia and hypertrophy give place to necrosis virus growth decreases as the cells available for multiplication diminish. From then on destruction of virus and transportation of virus to other parts of the egg predominate over the production of new virus, and the overall infective titre in the cells drops.

SUMMARY.

The multiplication of herpes virus in the cells of the chorio-allantois of the developing chick embryo and in tissue culture of the Maitland type has been studied. The virus was shown in both cases to be rapidly absorbed by the cells, to pass through a period during which little infective virus could be detected, followed by a period of rapid increase of infective virus which continued, at first step-wise, for 48 hr. in the developing chick embryo and for 96 hr. in tissue culture, at which times maximum amounts of virus were found. In the chorio-allantoic membrane, subsequent multiplication cycles were shorter than the primary one, suggesting that the hyperplastic tissue was a better medium for virus production than the normal. A tentative picture of the development of herpes simplex virus is given.

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INTERFERENCE IMMUNITY PRODUCED BY PERTUSSIS VACCINE TO PERTUSSIS INFECTION IN MICE.

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Received for publication August 24, 1954.

WE have previously reported (Evans and Perkins, 1954) that a single intraperitoneal injection of pertussis vaccine produces in mice an early immunity to intracerebral infection with *Haemophilus pertussis*. This effect appeared to be specific for pertussis vaccine since it was not produced by a number of other antigenic preparations. The rapidity with which the immunity developed and the fact that it was independent of circulating specific antibodies suggested that the acquired resistance was of a type for which serological mechanisms were not responsible. The possibility was considered that the effect might be analogous to the " interference phenomenon " which has been shown to occur between inactivated and active virus of the same strain (Henle, 1950). This possibility has now been investigated by studying the protective action of pertussis vaccine when given to mice intracerebrally at the site of infection.

MATERIALS AND METHODS.

Vaccines.—Three of the pertussis vaccines, V12, V17 and YPS, which we used in our previous study were tested. Vaccines V12 and YPS were whole bacterial suspensions each containing 20×10^9 organisms/ml. (U.S. National Institutes of Health opacity standard). Vaccine V12 was killed by 1/10,000 thiomersalate and YPS by heating at 55° for ½ hr. Vaccine V17 was a cell-free bacterial extract prepared by Pillemer, Blum and Lepow (1954) by the method they described and which they have called SPA.

Inoculation of mice.—Female white mice with an average weight of 16 g. were used. They were injected intracerebrally with a challenge suspension of virulent *H. pertussis* according to the method described by Kendrick, Eldering, Dixon and Misner (1947) using strain No. 18/323 which they recommended. The challenge suspensions were prepared by suspending, to the desired concentration, the 24 hr. growth from Bordet-Gengou medium in casein hydrolysate. Each challenge suspension was titrated by injecting 5 groups, each of 15 mice, with graded dilutions and the number of LD_{50} contained in the challenge dose was calculated by the method of Reed and Muench (1938) from the number of deaths occurring in each group within 21 days after injection. With the strain of mice used, the LD_{50} of the challenge suspensions lay between 1000 and 2000 organisms (U.S. opacity standard).

Vaccine suitably diluted in casein hydrolysate was also injected intracerebrally into mice, either mixed with, or 3 hr. before the challenge suspension. When vaccine and challenge suspension were given together, the total inoculum was 0·03 ml., and when given separately, each inoculum was 0·03 ml. All intracerebral injections were made into the left cerebral hemisphere. It was found that mice were able to withstand 2 intracerebral injections into the same site and only on rare occasions did an early non-specific death occur. The mice were observed for 21 days after injection and the number of deaths and times to death recorded.

RESULTS.

Protective Effect of Vaccine Mixed with Challenge Dose.

Each of the 3 vaccines was tested for its ability to protect mice against infection when given intracerebrally together with the challenge dose. The results of a test

with vaccine V12 in which the challenge dose contained 40 LD_{50} are given in Fig. 1. In this test, 4 groups, each of 15 mice, were used. Three of the groups were each injected with the challenge dose suspended in vaccine diluted 1/3, 1/30 and 1/300 respectively and the fourth group with the challenge dose only. The doses of vaccine corresponding to the 3 dilutions contained 2×10^8, 2×10^7 and 2×10^6 organisms respectively. All the mice in the control group died by the 11th day after injection. Evidence of protection was shown by the group which received vaccine diluted 1/3 ; 9 of the 15 mice were alive on the 11th day and 7 on the 21st day after injection. There was also evidence of protection, although not so marked, with the groups which received vaccine diluted 1/30 ; 7 of the 15 mice were alive on the 11th day and 4 on the 21st day. Little or no protection, however, was given by vaccine diluted 1/300 ; in the group injected with this dilution, 14 of the mice died at about the same average time as those in the control group and only one mouse survived for 21 days.

The protective effect of vaccine V12, when given mixed with the challenge dose, was also evident from a second test (Fig. 2) in which the challenge dose contained approximately only 1 LD_{50}. Two groups each of 15 mice were used, one group receiving the challenge dose suspended in vaccine diluted 1/100 ; each mouse thus received 6×10^6 organisms of vaccine, and the other group the challenge dose only. Of the 15 mice in the group receiving vaccine, all survived the 21-day period except one which died on the 15th day after injection. Of the 15 mice in the control group, 7 died and all deaths occurred before the 13th day.

Similar tests were made with the other bacterial vaccine YPS and also with the bacterial-extract vaccine V17. The results obtained in one test with each vaccine are shown in Table I together with those obtained with V12. It is evident that each vaccine gave definite protection especially when given in dilutions of 1/3 and 1/30.

The protective property of YPS and V17 was also demonstrated in tests using a range of different challenge doses. Three series of the same 4 graded dilutions of a challenge suspension were made, one in a 1/30 dilution of YPS, a second in a 1/30 dilution of V17 and a third, the control series, in casein hydrolysate. Each dilution was injected intracerebrally into a group of 15 mice. The results are shown in Table II and it is evident that with each of the 4 challenge doses, which ranged from 0·5 to 150 LD_{50}, the two vaccines gave definite protection.

Tests were made which showed that the property of the vaccines to protect mice against infection was not due to an effect of the vaccines on the viability of the organisms *in vitro* before injection. A series of 10-fold dilutions of a challenge suspension was made in each of the three vaccines diluted 1/3 and also a control series in casein hydrolysate. The dilutions were left at room temperature for 1 hr. when plate counts were made in duplicate on Bordet-Gengou medium by the method of Miles and Misra (1938). There was no significant difference in the results obtained with each of the four counts, indicating that there was no effect on the viability of *H. pertussis* by any of the three vaccines.

Protective Effect of Vaccine Given Three Hours Before Challenge Dose.

Two of the vaccines, V12 and V17, were tested for their ability to protect mice against infection when given intracerebrally 3 hr. before the challenge dose. The results of two tests with the whole bacterial vaccine V12 are given in Fig. 3

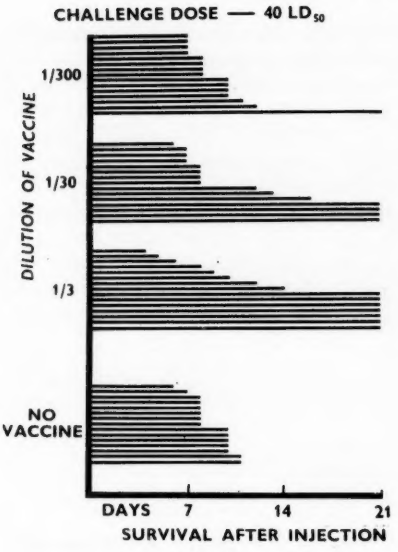

FIG. 1.

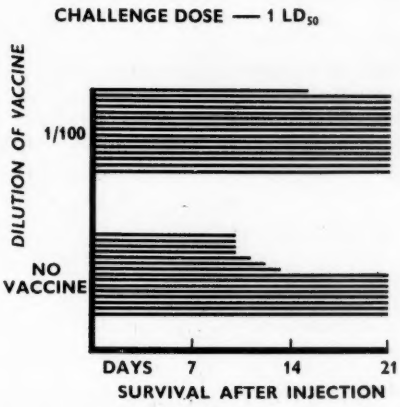

FIG. 2.

FIG. 1 and 2.—The protection of mice with whole bacterial pertussis vaccine V12 injected intracerebrally mixed with challenge dose of *H. pertussis*.

In these Figures and in Fig. 3 and 4, each horizontal line represents the duration of survival of one mouse; mice surviving for 21 days were killed.

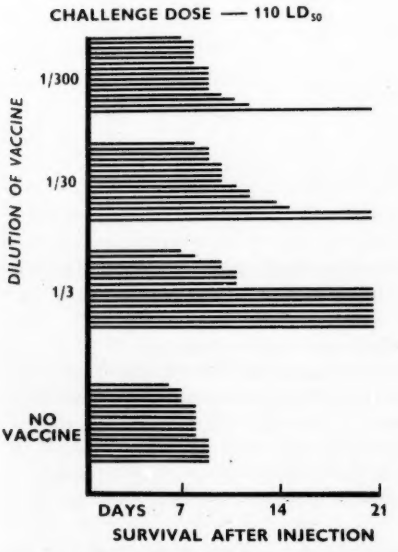

FIG. 3.

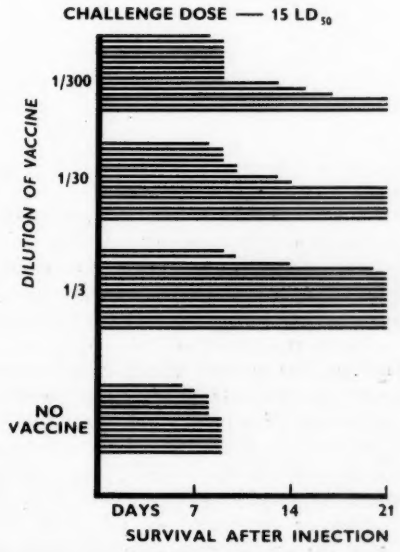

FIG. 4.

FIG. 3 and 4.—The protection of mice with whole bacterial pertussis vaccine V12 injected intracerebrally three hours before challenge dose of *H. pertussis*.

TABLE I.—*Protective Effect of Pertussis Vaccine when given Intracerebrally together with Challenge Dose of* H. pertussis.

Pertussis vaccine.	Dose of vaccine (dilution).	Challenge dose of *H. pertussis* (LD_{50})	No. of mice used.	No. of mice alive at— 10 days.	No. of mice alive at— 21 days.	Percentage of mice alive at 21 days.
V12	1/3	40	15	9	7	47
	1/30		15	7	4	27
	1/300		15	3	1	7
	None		15	2	0	0
V12	1/100	1	15	15	14	93
	None		15	11	8	53
YPS	1/3	150	14	13	10	71
	1/30		15	7	5	33
	1/300		15	5	3	20
	None		15	0	0	0
V17	1/3	150	13	10	5	38
	1/30		14	6	4	29
	1/300		15	3	0	0
	None		15	0	0	0

TABLE II.—*Protective Effect of Pertussis Vaccine when given Intracerebrally together with the Challenge Dose of* H. pertussis.

Vaccine and dose (dilution).	Challenge dose *H. pertussis* (LD_{50}).	No. of mice used.	No. of mice alive at 10 days.	No. of mice alive at 21 days.	Percentage of mice alive at 21 days.
YPS 1/30	0·5	15	15	14	93
	3	15	15	12	80
	15	15	13	8	53
	150	15	11	4	27
V17 1/30	0·5	15	15	15	100
	3	15	14	11	73
	15	15	9	5	33
	150	15	7	5	33
None	0·5	15	12	10	67
	3	15	5	3	20
	15	15	5	0	0
	150	15	0	0	0

and 4. Four groups, each of 15 mice, were used for each test. Three of the groups were each injected with vaccine diluted 1/3, 1/30 and 1/300 respectively and 3 hr. later all four groups were challenged.

In the first of these tests (Fig. 3) the challenge dose contained 110 LD_{50} and killed all the control mice by the 9th day. Vaccine diluted 1/3 showed a protective effect since 13 of the 15 mice which received this dilution were alive on the 9th day and 8 were alive on the 21st day after injection. Vaccine diluted 1/30 also showed a protective effect; 11 of the 15 mice receiving this dilution were alive on the 9th day and 2 on the 21st day. Only a slight effect was evident in the group receiving vaccine diluted 1/300; 4 mice were alive on the 9th day and one on the 21st day.

In the second test with V12 (Fig. 4) the protective effect of vaccine was more definite, which was probably due to the fact that a smaller challenge dose of 15

LD_{50} was used. Of the group of 15 mice which received vaccine diluted 1/3, 14 were alive on the 9th day, when all the mice in the control group were dead, and 11 on the 21st day. Of the group which received a 1/30 dilution of vaccine, 11 of the 15 mice were alive on the 9th day and 7 on the 21st day. Vaccine diluted 1/300 also gave some protection ; 6 of the 15 mice which received this dilution were alive on the 12th day and 3 on the 21st day.

The bacterial-extract vaccine V17 also gave protection when given 3 hr. before the challenge dose. The results of two tests with this vaccine are given in Table III which includes those with V12. In the first test with V17 a high challenge dose containing 350 LD_{50} was used, yet in spite of this vaccine diluted 1/3 gave substantial protection. In the second test a similar result was obtained using vaccine diluted 1/30 followed by a challenge dose containing 35 LD_{50}.

TABLE III.—*Protective Effect of Pertussis Vaccine when given Intracerebrally Three Hours before Challenge Dose of* H. pertussis.

Vaccine	Dose of vaccine (dilution).	Challenge dose of *H. pertussis* (LD_{50}).	No. of mice used.	No. of mice alive at 10 days.	No. of mice alive at 21 days.	Percentage of mice alive at 21 days.
V12	1/3	110	15	11	8	53
	1/30		15	7	2	13
	1/300		15	3	1	7
	None		15	0	0	0
V12	1/3	15	15	13	11	73
	1/30		15	9	7	47
	1/300		15	6	3	20
	None		13	0	0	0
V17	1/3	350	11	7	5	45
	None		15	0	0	0
V17	1/30	35	14	8	5	36
	None		15	3	0	0

DISCUSSION.

These experiments have shown that pertussis vaccine in the form of either a bacterial suspension or a cell-free extract, is able to protect mice against fatal infection produced by an intracerebral challenge injection of virulent *H. pertussis* when given at the site of infection either with or a few hours before the challenge dose. We have not yet followed the fate of the infecting organisms in the brains of mice after injection, but we have shown that in a high proportion of protected mice the brains are free from viable *H. pertussis* when cultivated 28 days after challenge. It is therefore evident that pertussis vaccine, by some mechanism of interference, is able to influence the course of intracerebral *H. pertussis* infection and that this interference effect is not a function of the intact whole bacterial cell.

These results agree with our previous findings (Evans and Perkins, 1954), which showed that an intraperitoneal injection of pertussis vaccine into mice is able to produce within 5 hours a substantial immunity to intracerebral infection with *H. pertussis*, in the absence of detectable circulating specific antibodies. This early immunity is probably due to the same mechanism as that involved when

vaccine is injected locally at the site of infection. Its development, however, would naturally be slower than that after intracerebral injection, since it would depend upon the time taken for the interfering agent to reach the site of infection in sufficient concentration to affect the course of the disease. This hypothesis is supported by the fact that although immunity is evident in mice within 5 hours after intraperitoneal injection, it is not, as we have since shown, evident in mice which have received vaccine only a few minutes before challenge.

What mechanisms are involved in this interference phenomenon are not known, but it is reasonable to suggest that they may be similar to those occurring in the case of viruses where the cellular basis for interference is well established. Support for this suggestion is provided by the work of Dalldorf, Cohen and Coffey (1947) and Cohen (1953) who showed that mice were protected against infection following a small intracerebral challenge dose of *H. pertussis* by a prior intracerebral injection of certain viruses.

The early immunity produced by pertussis vaccine must be distinguished from that which develops later and is especially evident after repeated intraperitoneal injections. We have shown in a few preliminary experiments, using the whole bacterial vaccine V12, that the later immunity is of the antibody type and that serum from immunized mice is able to protect mice passively against intracerebral pertussis infection. It is not known, however, how soon after injection of vaccine the antibody type of immunity manifests itself or at what stage the interference effect begins to wane. It may in fact be difficult to discriminate between these two types of immunity, especially if the antigen responsible for producing the antibody type is also the responsible agent in the production of the early interference phenomenon. These are problems which are at present under investigation.

SUMMARY.

Pertussis vaccine, either in the form of a killed bacterial suspension or a cell-free extract, was shown to interfere with the development of fatal pertussis infection in the mouse. Mice were protected against infection following an intracerebral challenge injection of *H. pertussis* by an intracerebral injection of pertussis vaccine given at the same time or 3 hr. before the infecting challenge dose.

We wish to thank the Medical Research Council for samples of pertussis vaccines received from the Whooping Cough Immunization Committee and for a grant towards the expenses of this work.

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THE EFFECT OF TUBERCULIN ON THE RESPIRATORY RATE OF SKIN FROM SENSITISED GUINEA-PIGS.

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Received for publication September 16, 1954.

ONE of the classical distinctions usually drawn between the tuberculin or delayed type of hypersensitivity and the urticarial or immediate type of response is the fact that tuberculin has a cytotoxic action *in vitro* on the tissues of sensitised animals (Rich and Lewis, 1932 ; Moen and Swift, 1936). This has led to a widespread belief that the tuberculin reaction *in vivo* occurs as the result of a necrosing effect of tuberculin on the skin. A previous study however (Cruickshank, 1951) showed that sensitised skin slices cultivated *in vitro* by the method of Medawar (1948) showed no abnormality in the presence of tuberculin. Since then Baldridge and Kligman (1951) have failed to repeat Rich's findings and Everett, Livingood, Pomerat and Funan (1952) have shown that tuberculin and chemical sensitising agents have no toxic effects on tissue cultures of human epidermal cells from subjects showing sensitivity to skin tests.

Histological studies have recently led Gell and Hinde (1954) to advance the idea that the tuberculin reaction *in vivo* is essentially proliferative. *In vitro* however there is no histological evidence of proliferation of mesenchymal cells of the dermis. Since it was considered that histological examination of the skin after cultivation might not be a sufficiently sensitive method to reveal small changes of either a toxic or proliferative nature further experiments have been made in which the oxygen consumption of the skin cultures has been used as a measure of their biological activity.

With conventional apparatus for measuring oxygen consumption the period of observation is usually limited to some 4 hr. This is an insufficient period on which to base a decision concerning the effects of tuberculin, as, *in vivo*, the reaction does not apparently begin until 4 hr. have elapsed and is usually maximal at 24 hr. Accordingly a special differential capillary microrespirometer has been devised which will permit continuous observation of skin cultures for prolonged periods.

MATERIALS AND METHODS.

Apparatus.

The microrespirometer which is described in detail elsewhere (Cruickshank, 1955) consists of two chambers of the same size drilled in a brass block and communicating by means of a capillary of 0·4 mm. bore which is sealed in the top, which is also made of brass. In each chamber is placed a glass cup containing culture medium which consists of :

Serum (usually autologous)	0·5 ml.
Krebs-Ringer phosphate	0·30 ml.
Glucose 5 per cent	0·1 ml.
Streptomycin in distilled water (500 μgm./ml.) .	0·1 ml.

The carbon dioxide is absorbed from each chamber by means of filter paper soaked with 2 per cent KOH. An indicator droplet of specially prepared kerosene is placed in the capillary and its movement read on an attached plastic scale. In later models the capillaries were calibrated.

Thin skin slices cut free-hand from the dorsum of the guinea-pig ear are floated on the surface of the medium in one of the chambers, approximately 10 mg. initial wet weight being used. As O_2 is used and CO_2 absorbed the indicator droplet travels in the direction of the culture and the amount of O_2 used is given by the formula $\Delta^v{}_p = 2\,ad$, where

$\Delta^v{}_p$ = change in gas volume
a = cross-section area of capillary
d = distance of movement of the droplet.

A special tap is used for the admission of gases and liquids, in this instance tuberculin, to the cultures during the course of the experiments.

Animals.

White guinea-pigs were sensitised by a single intradermal injection of 1·0 mg. killed tubercle bacilli in liquid paraffin. Only the animals showing a high sensitivity were subsequently used. These gave reactions of 18–25 mm. in diameter to 1/100 O.T. which had a necrotic central area of about 7–10 mm. diameter.

Experimental method.

Each experiment was carried out with 4 respirometers, sufficient skin being obtainable from the ears of one guinea-pig. The weight of skin used in each respirometer was 6–10 mg. (initial wet weight). The respiratory rate of the skin slices in each of the respirometers was determined during a preliminary observation of 2 hr. Tuberculin was then added to both chambers of two respirometers and saline to both chambers of the other two. Two strengths of tuberculin were used, P.P.D. 0·04 mg./ml. and P.P.D. 0·16 mg./ml., 0·05 ml. being added to give final concentrations in the medium of 0·002 mg./ml. and 0·008 mg./ml. These correspond with concentrations of O.T. of 1/100 and 1/25 respectively.

After the addition of tuberculin, readings were continued for a further 2 hr. Observations were not made overnight but were continued for a further 2-hr. period the following morning. In the series of experiments the effects of tuberculin could thus be determined on the individual skin slice immediately after addition and 24 hr. later. Each determination was accompanied by a saline control. The experimental method effectively controlled the variations due to the state of the animal and to slice thickness, etc.

RESULTS.

Tables I and II show the results of 8 experiments, 4 on normal (Table I) and 4 on sensitised animals (Table II). Two experiments were discarded because of the development of a contamination in the medium due to a mould, *Absidia ramosa*.

TABLE I.—*The Effects of Tuberculin and of Saline on the Respiration of Normal Guinea-pig Skin.*

Animal No.	Slice No.	Treated *in vitro* with :	Oxygen uptake (μl./mg./hr.). (a) Initial.	(b) 2–4 hr.	Difference (b)–(a).	(c) 22–24 hr.	Difference (c)–(a).
I	1	P.P.D. 0·002 mg./ml.	1·05	1·06	+0·01	1·11	+0·06
	2	" " "	1·06	1·13	+0·07	1·11	+0·05
	3	Saline	1·11	1·15	+0·04	1·07	+0·04
	4	"	1·26	1·28	+0·02	1·23	−0·03
II	1	P.P.D. 0·002 mg./ml.	1·10	1·15	+0·05	1·11	+0·01
	2	" " "	1·05	1·15	+0·10	1·07	+0·02
	3	Saline	0·82	0·89	+0·07	0·92	+0·10
	4	"	1·03	1·04	+0·01	1·05	+0·02
III	1	P.P.D. 0·008 mg./ml.	1·12	1·14	+0·02	1·12	0
	2	" " "	1·10	1·16	+0·06	1·09	−0·01
	3	Saline	1·14	1·19	+0·05	1·17	+0·03
	4	"	1·28	1·36	+0·08	1·21	−0·07
IV	1	P.P.D. 0·008 mg./ml.	1·12	1·13	+0·01	1·09	−0·03
	2	" " "	1·02	1·03	+0·01	1·02	0
	3	Saline	1·17	1·22	+0·05	1·20	+0·03
	4	"	1·21	1·21	0	1·18	−0·03

TABLE II.—*The Effects of Tuberculin and of Saline on the Respiration of Sensitised Guinea-pig Skin.*

Animal No.	Slice No.	Treated *in vitro* with :	Oxygen uptake (μl./mg./hr.). (a) Initial.	(b) 2–4 hr.	Difference (b)–(a).	(c) 22–24 hr.	Difference (c)–(a).
I	1	P.P.D. 0·002 mg./ml.	1·06	1·12	+0·06	1·06	0
	2	" " "	1·13	1·15	+0·02	1·08	−0·05
	3	Saline	0·99	1·04	+0·05	1·06	+0·07
	4	"	1·11	1·16	+0·05	1·13	+0·02
II	1	P.P.D. 0·002 mg./ml.	1·23	1·26	+0·03	1·23	0
	2	" " "	1·22	1·22	0	1·25	+0·03
	3	Saline	1·28	1·26	−0·02	1·21	−0·07
	4	"	1·22	1·22	0	1·20	−0·02
III	1	P.P.D. 0·008 mg./ml.	0·99	0·99	0	0·99	0
	2	" " "	1·08	1·08	0	1·04	−0·04
	3	Saline	1·00	1·00	0	1·02	+0·02
	4	"	0·98	0·97	−0·01	1·00	+0·02
IV	1	P.P.D. 0·008 mg./ml.	0·95	0·99	+0·04	0·95	0
	2	" " "	1·09	1·09	0	1·13	+0·04
	3	Saline	0·99	0·98	−0·01	1·03	+0·04
	4	"	1·05	1·09	+0·05	1·13	+0·08

From Table I it can be derived that the mean respiratory rate of the skin slices before tuberculin was added was 1·08 μl./mg./hr. In the ensuing 2 hr. in the presence of tuberculin the mean rate was 1·12 μl./mg./hr. and 24 hr. later 1·09 μl./mg./hr. The corresponding figures for the effect of saline are 1·11, 1·15 and 1·11. It will be noted that there is a tendency for the respiration of both saline-treated and tuberculin-treated to increase slightly between the second and fourth hour, the mean increase being 0·04 μl./mg./hr. During this period the pH of the medium becomes more alkaline as CO_2 is given off from the serum. Experiments have shown that a rise of pH from 7·4–8·2 (the range occurring during this

period) is associated with an increased respiratory rate. By allowing a longer equilibration period in the second group of experiments (Table II) this rise was minimised.

The main criterion of the effects of tuberculin however is the comparison of the differences between the rates before treatment and after treatment with tuberculin and with saline. These differences are shown in Table III. Statistical testing fails to reveal any significant differences either immediately after tuberculin or 24 hr. later.

The pattern of results obtained with skin from sensitised animals follows closely that obtained with the unsensitised (Table II). No differences could be detected between the effects of saline and of tuberculin (Table III). It will be noted that the difference between the effects of saline and of tuberculin at 2–4 hr. is 0 and at 24 hr. is 0·01 μl./mg./hr. with a standard error of $\pm$0·02. It must be concluded that any difference which the experimental method has failed to detect is less than $\pm$ 2 per cent.

TABLE III.—*Mean Differences in Rates (μl./mg./hr.) of Oxygen Uptake by Guinea-pig Skin.*

		P.P.D. (both strengths).	Saline.
Normal guinea-pigs	at 2– 4 hr.	0·04 S.E. $\pm$0·01	0·04 S.E. $\pm$0·01
	„ 22–24 „	0·01 „ $\pm$0·01	0 „ $\pm$0·02
Sensitised „	at 2– 4 „	0·01 „ $\pm$0·01	0·01 „ $\pm$0·01
	„ 22–24 „	0 „ $\pm$0·01	0·01 „ $\pm$0·02

DISCUSSION.

These experiments confirm the tissue culture studies previously reported and show that tuberculin does not affect the rate of oxygen uptake of sensitised skin *in vitro*. The slices contained both epidermal and mesenchymal cells, the former contributing the greater share of the respiratory activity. It is likely however that sufficient dermis is present to ensure the detection of all but the slightest of toxic or stimulating effects. As the most convincing demonstrations of cytotoxicity of tuberculin on sensitised cells *in vitro* have been made with mixed cultures of polymorphonuclear leucocytes, lymphocytes and mononuclear cells from spleen or lymph gland (Rich and Lewis, 1932 ; Moen and Swift, 1936), it would seem that the presence of such cells in the circulation of the area injected is necessary for the initiation of the tuberculin reaction *in vivo*. Further evidence that circulating mesenchymal cells are necessary is provided by the recent studies of Pepys (1953), who showed that the size of the tuberculin reaction could be varied by altering the local blood flow to the skin by adrenalin and histamine. The fact that circulating leucocytes are lysed by tuberculin has been demonstrated by Favour (1947). The outward manifestations of the tuberculin reaction are clearly vascular and possibly secondary to the cellular " infiltration". Whether the cellular infiltration represents the aggregation of antibody-carrying cells or is a reaction of the local tissue cells is not clear. The experiments reported here suggest however that cells carried by the circulation are necessary for the development of any local reaction.

SUMMARY.

The effect of tuberculin on the oxygen uptake of skin from normal and from sensitised guinea-pigs has been investigated. No alteration in the respiratory rate could be detected in either.

I am indebted to Mr. J. R. Cooper, F.I.M.L.T. for his valuable assistance, and to Professor J. R. Squire, Leith Professor of Experimental Pathology, University of Birmingham, and Honorary Director of this Unit, for his continued interest and advice.

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SURVEY OF PAPERS.

JONES, YOLANDE CARTER and RANKIN have inoculated guinea-pigs repeatedly with polysaccharides extracted by several methods from *Kl. pneumoniae*. One of these was often lethal, and sensitized the animal, as did the bacteria themselves, to a further injection. This "anaphylactogenic" property was lacking in polysaccharides which elicited joint lesions. Lesions of cardiac valves were also produced. These differences of behaviour were not accounted for by chemical analytical findings (p. 519).

ROWLEY has attempted to account for the differences in pathogenicity for mice found among various strains of *Bact. coli*. These differences were found to be independent of sensitivity to an amino acid (serine), and to be unrelated to demonstrable antigenic structure or to the capacity to ferment sugars. Virulent strains were shown to be more resistant *in vitro* to the bactericidal action of serum containing complement, and he postulates that this resistance is an important factor in determining virulence (p. 528).

KROHN has shown that although cortisone administered at the same time does not affect the fate of second set skin homografts, it prolongs their survival if administration is begun considerably earlier than the second grafting operation. It is considered to act by interfering with the immune response (p. 539).

O'CONNOR has produced small areas of necrosis in the descending colon of the mouse by the application of solid silver nitrate through a tube inserted *per anum*, under direct observation through an abdominal incision. Repair was studied histologically at intervals varying from a few hours to 1 month. The results suggest that if the bases of the mucous glands survived the application, full regeneration followed. If the necrosis extended more deeply, the granulation tissue filling the gap became only partly covered by an ingrowth of epithelium in which no mucous glands developed (p. 545).

CORA P. CHERRY, BLAND, CONSTABLE, HUGHES and HARRIS, having observed that continued administration of ACTH or cortisone to guinea-pigs is followed by an increase in liver weight, have investigated the basis of this, and shown it to be due to an increase in size of individual liver cells. The enlargement affects the cytoplasm only, and is greater after cortisone than after ACTH (p. 551).

SELBIE and O'GRADY describe a technique for the production of a localised and measurable tuberculous abscess in the thigh of the mouse. This lesion, which does not noticeably interfere with the general health of the animal, resolves, leaving the limb apparently normal. Evidence is put forward in support of the view that in the mouse hypersensitivity and immunity are separate phenomena, hypersensitivity existing from a very early stage in the infection, and immunity appearing during the third week (p. 556).

ROSA M. CAMPBELL, GILLIAN SHARP, BOYNE and CUTHBERTSON have used urinary nitrogen excretion as a measure of metabolic response to injury, and found the increase in it resulting from fracture of a femur to be of the same order as that following implantation of a tablet of cortisone. Part of the response to the latter is due to the incision. Fracture produced no response in adrenalectomized animals, but a response was obtained when they were maintained on cortisone. The relationship between the response the basal nitrogen excretion level and food intake is also analysed (p. 566).

BRANDES and BOURNE have studied, by histochemical methods, the amount and distribution of various enzymes and of P.A.S.-positive substances in the prostate of normal and castrated mice. Acid phosphatase was found within epithelial cells and alkaline phosphatase

in the stroma and basement membranes. These enzymes and P.A.S.-reactive material were reduced after castration (an effect reversed by testosterone) or after implantation of stilboestrol (p. 577).

MARGARET EDNEY and LIM have investigated the stability and purity of a recombinant (neuro-MEL) strain of influenza virus by repeated study of various marker characters, including infectivity for the mouse brain, during passage in eggs. These properties remained constant through four transfers at high dilution. From eggs infected with a limiting dilution, pure recombinant virus was again obtained (p. 589).

MODI and TOBIN have studied the growth of herpes virus in the chorio-allantoic membrane of the chick embryo and in minced chick embryo *in vitro*. They found an early disappearance of the infective virus followed by a step-wise increase reaching a maximum at 48 hr. in the egg and later in the cultures. Complement-fixing antigen appeared later than infectivity and was not proportional to the infective titre. A tentative picture of the development of herpes virus is given (p. 595).

EVANS and PERKINS, in continuation of previous work, have shown that pertussis vaccine given at the site of infection will protect mice against a fatal intracerebral dose of *H. pertussis*. The vaccine may be given with the challenge dose or a few hours before it, and it is suggested that the immune state so rapidly produced may resemble that due to the interference phenomenon which has been described for virus infections (p. 603).

CRUICKSHANK has shown by means of a microrespirometer permitting observations over long periods that tuberculin does not affect oxygen uptake by slices of skin from sensitized guinea-pigs (p. 609).

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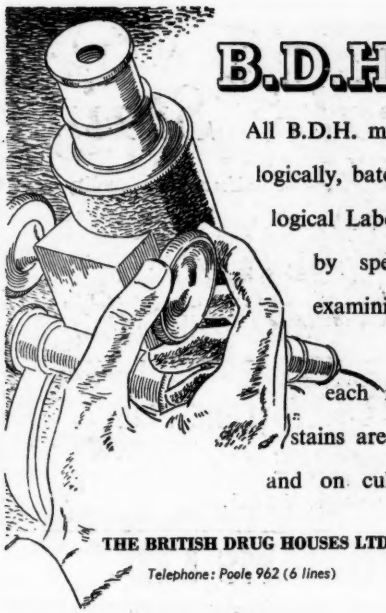

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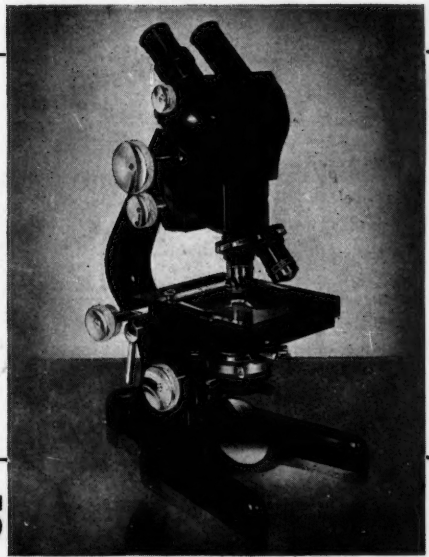

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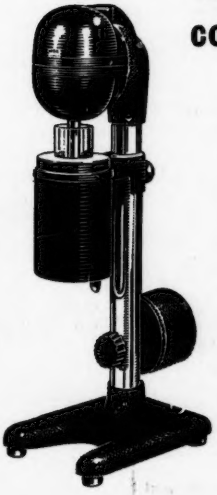

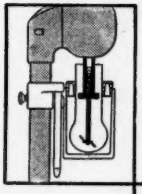

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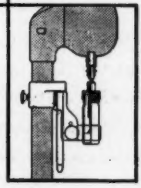

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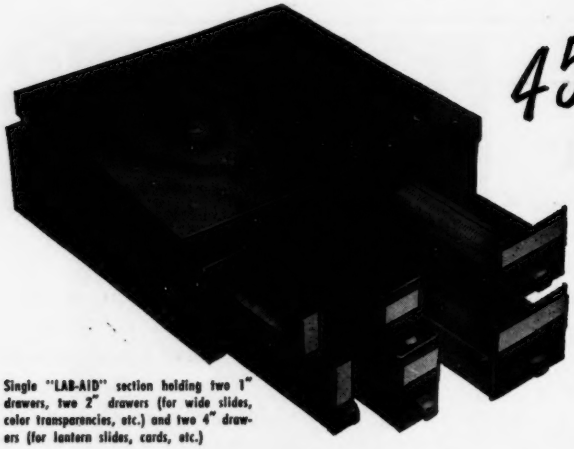

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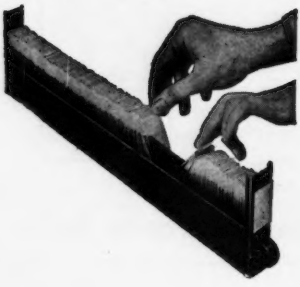

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